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**“REGULATION OF p14ARF TUMOR
SUPPRESSOR ACTIVITIES AND
FUNCTIONS”**

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**REGULATION OF p14ARF TUMOR SUPPRESSOR
ACTIVITIES AND FUNCTIONS**

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LIST OF PUBLICATIONS RELATED TO THE THESIS.

Vivo M, **Ranieri M**, Sansone F, Santoriello C, Calogero RA, Calabrò V, Pollice A, La Mantia G. “Mimicking p14ARF phosphorylation influences its ability to restrain cell proliferation”.

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ABSTRACT

The ARF protein is encoded by the alternative reading frame of the INK4a locus, one of the most frequent sites of genetic loss in human cancers. The ARF tumor suppressor function, as a sensor of hyper-proliferative stimuli, is to restrict cell proliferation through both p53-dependent and independent pathways. However, few studies started to address the possibility that ARF might promote the survival of subsets of tumors.

In the past years, intensive studies have been focused not only on ARF activation at transcriptional level but also on the mechanisms regulating ARF protein turnover. Here we show that p14ARF is a PKC target. In fact, PKC activation mediates phosphorylation and stabilization of ARF endogenously expressed protein. Moreover, a phosphomimetic ARF mutant accumulates in the cytoplasm and, interestingly, despite the ability to stabilize p53 and bind MDM2, is not able to efficiently block cell growth in both human and mouse cell lines. These data could be interpreted as a way of cancer cells to escape ARF surveillance in tumorigenesis, but they might also indicate that ARF phosphorylation in tumor cells could be a mean to sustain tumor progression by conferring pro-survival properties to the cells. We thus explored the role of endogenously expressed ARF protein in some tumor and stabilized cell lines by knocking down ARF expression by siRNA. Unspectedly, we observed a block of cell proliferation and induction of DAPK mediated p53-dependent apoptosis in ARF depleted Hela and HaCat cells. Our results suggest that ARF might have a pro-survival role depending on cellular context.

1. BACKGROUND

The ARF protein is encoded by the **A**lternative **R**eadin**G** **F**rame of the INK4a locus, one of the most frequent sites of genetic loss in human cancers after p53 locus (Hainaut et al, 1997; Hall and Peters, 1996; Muniz et al, 2011).

A direct contribution of ARF to tumor formation has been documented using genetic analysis of tumors, molecular and cell biology methods and animal models (Muniz et al, 2011; Shimizu et al, 2010).

It has been reported that ARF-null mice are highly cancer prone. Particularly, ARF knock out mice die after 1 year from spontaneous tumor development, with a mean survival latency of 38 weeks. Moreover, heterozygous mice also develop tumors after a longer latency than ARF-null mice.

The 43% of tumors observed in ARF-null mice were sarcomas. In particular, a malignant osteogenic sarcoma with pulmonary metastasis and an angiogenic sarcoma metastatic to the liver were observed. The others tumors observed in ARF-null mice were 29% of lymphomas, 17% of carcinomas and 11% of gliomas (Kamijo et al, 1999).

Although alterations of INK4a-ARF locus are not common in humans, they were found in roughly 30% of human tumors such as glioblastoma, melanoma, pancreatic adenocarcinoma (Maggi et al, 2014; Sharpless and DePinho, 1999; Sherr, 1998). In the majority of the cases all three proteins of the INK4b-ARF-INK4a locus are lost, making difficult to determine their individual roles in human tumor suppression. In particular, many mutations within exon 2 that affect both ARF and p16Ink4a are found in cancers ((del Arroyo and Peters, 2005; Gardie et al, 1998; Rizos et al, 2001; Rutter et al, 2003; Zhang and Xiong, 1999). However, there are specific examples where only ARF appears to be affected in human cancer, and these cases appear to be most common in melanoma patients (Randerson-Moor et al, 2001). In addition to melanoma cases, nine of fifty glioblastoma patients have a specific deletion of ARF

(Nakamura et al, 2001). Furthermore, the ARF promoter contains a CpG island, and ARF expression is frequently downregulated by promoter methylation (Maggi et al, 2014).

Taken together, this collective wealth of evidence clearly demonstrates the importance of ARF tumor suppression in human cancers.

In contrast to its oncosuppressive role, there is a significant fraction of human tumors (prostate and lymphoma cancers) where ARF is overexpressed, suggesting that depending on the cellular context ARF may possess a pro-survival function (Basso et al, 2005; Chen et al, 2010; Humbey et al, 2008; Xie et al, 2014). In the last few years, in fact, different laboratories have started to elucidate this hypothesis by analyzing ARF role in several cellular contexts. In particular, it has been reported that loss of p19ARF fails to promote prostate cancer driven by loss of Pten (Chen et al, 2009). In fact, the histopathological analysis of prostates derived from p19ARF^{-/-}; Pten^{-/-} double mutant mice showed a decreased percentage of prostatic intraepithelial neoplasia compared to Pten^{-/-} mice. They also observed that ARF pro-survival role was cell context specific. In fact, ARF loss in mouse embryo fibroblasts resulted in the increase of cell proliferation (Chen et al, 2009). Furthermore, ARF silencing in Myc-driven lymphoma cells, containing mutant p53, impedes their survival under nutrient-starved condition and their development as tumor in xenograft assays (Humbey et al, 2008). All together these observations led to the novel hypothesis that ARF might have tumor-type specific survival functions.

1.1 THE HUMAN INK4/ARF LOCUS ENCODES p14ARF PROTEIN.

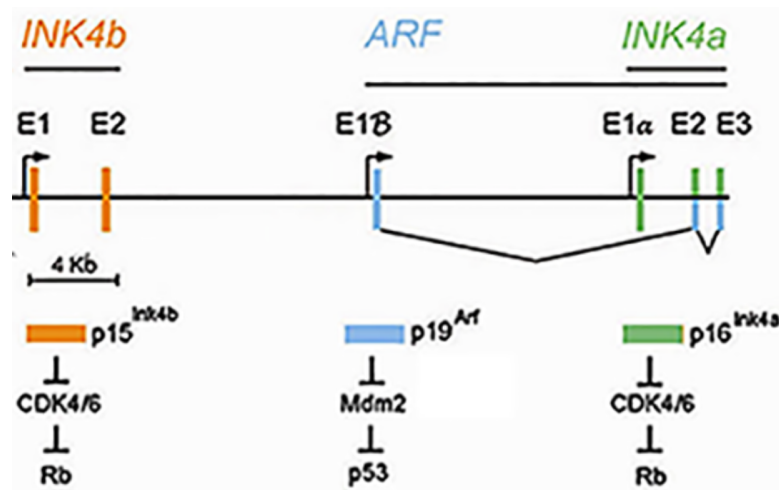
The ARF-INK4a locus located on human chromosome 9q21, encodes two completely unrelated proteins, p16INK4a and p14ARF, both potent inhibitors of cell proliferation. The mechanism by which these two proteins are produced is quite unusual. Each transcripts has a specific 5' exon, E1 α or E1 β for INK4a and ARF respectively, that are spliced to a common exon 2. This exon contains two overlapped ORFs, therefore the two proteins encoded share no amino acid sequence identity (Figure 1).

The alpha transcript, encoding p16INK4a, was the first to be identified, so the protein produced by the alternative beta transcript was named ARF, where ARF stands for alternative reading frame. In addition, it has been found by characterization of the region of human chromosome 9 (chromosome 4 in mouse) the presence of the INK4b gene (also known as CDKN2B) that is generated from the tandem duplication of INK4 gene and encodes the kinase inhibitor p15^{INK4b} (Sharpless and DePinho, 1999). Both p16^{INK4a} and p15^{INK4b} proteins interact and inhibit the kinase activity of cyclin dependent kinases 4 and 6 (CDKs) which in turn affect Rb pathway and led to E2F repression and growth arrest. Moreover, their binding prevents the interaction of the CDKs 4 and 6 with the D-type cyclins, required for their catalytic activity and for the cell cycle progression from G1 to S phase (Russo et al, 1998; Serrano et al, 1993; Sherr, 2006).

The β transcript results in a polypeptide of 132 amino acids and 14 KDa named p14ARF. The mouse β mRNA is translated in a 169 amino acids polypeptide named p19ARF (19KDa). Both mouse and human ARF regulate the induction of cell cycle arrest and/or apoptosis by p53-dependent and independent pathways (Ozenne et al, 2010; Sherr, 2006). Mouse and human ARF polypeptides are 45% identical through their exon 1 β segments and 50% identical overall. By comparison, mouse and human INK4a exon 1 α segments are 72% identical, and the p16^{INK4a} proteins share 65% identity overall

(Korgaonkar et al, 2005).

ARF is an unstructured protein, highly basic (22% arginine) and highly insoluble (DiGiammarino et al, 2001). In addition, it is well known that ARF needs to interact with other proteins to bring its charge to a more neutral pH and to explicate its functions (Maggi et al, 2014; Ozenne et al, 2010; Sherr, 2006).



Sherr C. J. 2012

Figure 1. View of the INK4-ARF locus.

The ARF-INK4 locus is located on human chromosome 9q21.

The alpha transcript consists of exon 1 alpha, exons 2 and 3, and encodes p16^{INK4a} protein. The beta transcript generated from an alternative ORF, consists of exon1 beta, exon 2 and exon 3 and encodes p19Arf protein (p14ARF in humans). The INK4b locus encodes p15^{INK4a} protein. The p14 ARF protein inhibits Mdm2 with consequential activation of p53. The p15^{INK4b} and p16^{INK4a} proteins are shown to inhibit CDK4/6 to maintain Rb in its growth-suppressive mode (Sherr, 2012).

1.2 THE ONCOSUPPRESSIVE ROLE OF p14ARF PROTEIN.

One of the most well defined function of ARF protein is to suppress aberrant cell growth in response to oncogene insult by activating the transcription factor p53 that trigger the expression of many apoptosis inducers and cell cycle inhibitory genes (Ozenne et al, 2010).

P53 mutation is the most frequent genetic alteration in human cancers. In particular, it has been shown that overexpression of p53 in both normal and neoplastic proliferating cells results in a context-dependent cell cycle arrest or apoptosis induction. It is well known that p53, for its important role, is subjected to stringent multi-level regulation. It has been widely reported that MDM2 interacts with p53, blocks p53-mediated transactivation and targets the p53 protein for rapid degradation (Chen et al, 1995; Haupt et al, 1997; Kubbutat et al, 1997; Levine, 1997). Pomerantz and colleagues (1998) reported that p19ARF protein is involved in p53 regulation; in fact, by coimmunoprecipitation analysis they found that ARF was able to interact with the C-terminal domain of MDM2 and with p53 as a multiprotein complex *in vivo*. In addition to the induction of its transactivation activity, the restoration of p53 levels was also observed when ARF was expressed together with p53 and MDM2. This latter effect was due to, the reduction of the p53 ubiquitinated forms and thus the reduction of proteasome mediated degradation (Pomerantz et al, 1998). By using deletion mutants of ARF protein (able or not to localize in the nucleolus) it has been shown that both binding to MDM2 and the localization of ARF protein in the nucleolus are necessary for ARF-induced p53 stabilization, p53 activation and cell cycle arrest. In particular, the interaction between ARF and MDM2 sequestered MDM2 in the granular region of the nucleolus, a membrane-less dynamic subnuclear organelle where ARF typically resides (Weber et al, 1999). This underline that the nucleolar compartmentalization of the ARF-MDM2 complex is important for the ability of both mouse and human ARFs to inhibit the cell cycle progression. This

localization depends from the ARF NoLS and from a sequence inside the C-terminal RING domain of MDM2 that appear unmasked upon ARF binding (Weber et al, 2000). In fact, it is known that MDM2 can shuttle between the nucleoplasm and the nucleolus in an ARF-dependent manner (Freedman and Levine, 1998; Roth et al, 1998; Tao and Levine, 1999).

Although ARF is undoubtedly a critical component of the p53 pathway, there are some evidences that ARF has also the ability to restrain cell growth independently of p53. Mice lacking ARF, p53 and MDM2 are more tumor prone than those lacking only p53 and MDM2. Furthermore, ARF^{-/-} and ARF^{+/-} mice develop a broader spectrum of tumors than p53-null mice. In line with this Weber and colleagues (2000) showed that ARF overexpression can induce a G1 arrest in cells lacking p53. In particular, in cells deficient for ARF/p53/MDM2 (derived from triple knockout or TKO mice), they observed that the reintroduction of wild type ARF was able to prevent S phase entry and/or trigger apoptosis by mechanisms that did not require the expression of wild-type p53 protein. They also demonstrated a significant reduction of colony formation in ARF infected TKO mice. In addition, it has also been reported that p14ARF induces cell cycle arrest in a p53-independent manner in human lung tumor cells (Eymin et al, 2003). In particular, p14ARF expression determined a G2 arrest followed by apoptosis both in “in vitro” and “in vivo” models. In this latter case, upon p14ARF overexpression a decrease of tumor growth and induction of lung tumors regression was observed in xenograft models.

Taken together these results support a role of ARF in mediating p53-independent tumor suppressive functions and suggest that ARF also acts independently of the Mdm2-p53 axis in tumor surveillance.

In line with this, it has been reported that ARF can interact with a multitude of different cellular partners: proteins involved in transcriptional control (E2Fs, DP1, p63, c-Myc, Hif1 α , nucleolar proteins such as nucleophosmin (NPM/B23), viral proteins (HIV-1Tat), mitochondrial protein (p32) and many

others other (Figure 2). The variety of the ARF interactors strongly suggested that ARF has a wider role to protect the cells upon different types of insults. In particular, for some targets, ARF interaction causes alteration of stability. For example, B23/NPM and E2F become degraded by the proteasome in an ubiquitin-dependent manner, while the CtBP2 antiapoptotic transcriptional co-repressor and HIV-1 Tat become degraded by the proteasome in a ubiquitin-independent manner. Other targets change their localization like E2Fs, c-Myc, Foxm1B, MDM2, ATR, DP-1, Hif1a upon ARF expression. Only few others, like Tip60, Topo I and COMMD1 become activated or stabilized (Pollice et al, 2008). Moreover, it has been reported that p32 protein, localized in the mitochondrial matrix and involved in cellular apoptotic response, is able to interact with ARF and to determine ARF's mitochondrial localization (Itahana and Zhang, 2008).

In addition, it has been reported from several laboratories that ARF is able to promote sumoylation of some of its interactors. Sumoylation is a post-translational modification that provide a covalent addition of the small ubiquitin-like modifiers (SUMO1, SUMO2, SUMO3) to ϵ -amino group of lysine residues in the target proteins that have the specific consensus sequence Ψ KXE (Ψ = hydrophobic amino acid). This modification can affects an high variety of phenomena such as protein stability, transport, modulation of gene expression (up-regulation or down-regulation), ubiquitination, DNA repair, and centromeric chromatid cohesion (Tago et al, 2005). In particular, it has been reported that ARF interacts with the Myc-associated zinc finger protein Miz1 and, by inducing its sumoylation, facilitates the assembly of the Myc-Miz1 complex that provide to the switch from G1 arrest to apoptosis (Herkert et al, 2010). Because transcription factors sumoylation can mediate the recruitment of corepressors, they hypothesize that sumoylation of Miz1 enhances repression by the Myc-Miz1 complex.

Moreover, it has been shown that ARF can induce sumoylation of both MDM2 and nucleophosmin (NPM/B23). It has been demonstrated that this ARF

function can be explicated only by direct interaction between ARF and its partners, and also in a p53-independent manner (Tago et al, 2005). In line with this, it has been shown an increase of sumoylated forms of HDM2, E2F-1, HIF-1 α , TBP-1 and p120E4F when p14ARF is expressed (Sherr et al, 2005). Although the precise mechanism underlying this ARF function is currently unknown it has been suggested that ARF explicates this function through a direct interaction with the sumo-conjugating enzyme Ubc9. Additionally, it has been reported that ARF also inhibits the function of a de-sumoylating protein, SENP3 (Haindl et al, 2008).

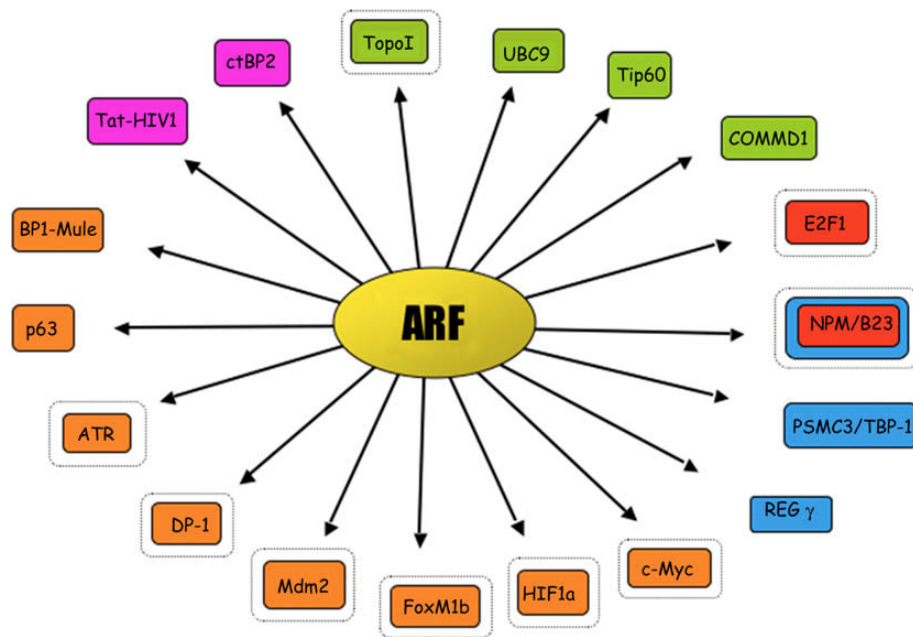
Although ARF involvement in the sumoylation process is well documented, the biological meaning of ARF mediated sumoylation is still unclear.

It has been reported that ARF interacts with p63, a transcription factor involved in the development of skin and limb, and induces its sumoylation both in HaCat cells (spontaneously immortalized keratinocytes) and in human primary keratinocytes. Vivo and colleagues (2009) demonstrated that ARF induce p63 sumoylation and proteasome mediated degradation. In particular, they also showed an increase of both ARF and SUMO2/3 protein levels and the concomitant decrease of p63 after induction of differentiation program in HaCat cells (Vivo et al, 2009). These results suggest that not only ARF-mediated sumoylation occurring during skin differentiation, but also that ARF can restrict cell proliferation in more physiological processes.

As ARF is mainly localized in the nucleolus this led to the hypothesis that it might play a role in the ribosomal biogenesis (Saporita et al, 2007). ARF implication in these process is still matter of debate. In mammalian cells a 47S rRNA precursor transcribed by RNA polymerase I (Pol I) is sequentially cleaved to yield the mature 28S and 5.8S rRNAs that form the core of the large 60S ribosomal subunit, as well as the 18S rRNA of the smaller 40S subunit. It has been shown that in ARF inducible cells, the formation of 28S and 18S rRNA was inhibited upon p19ARF induction (Sugimoto et al, 2003).

Moreover, by using a ChIP approach it has been reported that p14ARF can specifically associate with a chromatin fraction that contains the rRNA gene promoter suggesting that p14ARF can play a role in modulating rRNA transcription (Ayrault et al, 2004).

Other experimental evidences that confirm a role of ARF in ribosome biogenesis derived from the fact that ARF is able to inactivate nucleophosmin (NPM), a key player in ribosome biogenesis (Saporita et al, 2007). In fact, there are data that show inhibition of rRNA processing following the downregulation of NPM mRNA. In addition, it has been shown by blocking NPM nucleo-cytoplasmic shuttling that ARF is able to interfere with the ribosome export resulting in a delayed rRNA transcription and processing (Sherr, 2006).



Pollice et al, 2008

Figure 2. Schematic view of ARF binding partners.

Orange is for partners whose activity is blocked by ARF. Red is for partners that are induced to proteasome and ubiquitin dependent degradation by ARF. Pink is for partners that are induced to proteasome and ubiquitin independent degradation by ARF. Green is for partners whose activity or stability are positively regulated by ARF. Blue is for partners that regulate ARF protein turnover. A second black circle indicate nucleolar sequestration (Pollice et al, 2008).

1.3 ARF AS AN ONCOGENE: A NEW ROLE.

Despite its established role as tumor suppressor, ARF is overexpressed in a significant fraction of human tumors (Basso et al, 2005). For several years it has been taken for granted that ARF was unfunctional in these tumors. Interestingly, Chen and colleagues (2009) showed that loss of p19ARF in prostate epithelium does not accelerate but rather partially inhibits the prostate cancer phenotype of Pten deficient mice. In fact, they reported that there was a decrease in the incidence of prostatic intraepithelial neoplasia in the ventral prostate of double-null mice (Pten^{-/-} p19ARF^{-/-}) compared with Pten^{-/-} mice. Furthermore, using tissue microarrays of human prostate tumor samples they found that p14ARF loss was very rare in human prostate cancer and that the higher levels of p14ARF were correlated with disease aggressiveness. On the other hand, they instead observed an increase in cell proliferation and cell transformation in mouse embryo fibroblasts (MEFs) lacking both Pten and p19ARF by using a soft agar transformation assay. On the basis of these data they concluded that upon Pten loss and p19ARF inactivation there are different consequences in prostate cancer and MEFs that are independent of the p53 pathway and are context dependent (Chen et al, 2009).

In addition, last year Xie and colleagues (2014) reported that deletion of p19ARF remarkably resulted in growth suppression of prostate tumors of Pten/Trp53/p19ARF triple knockout mice. They found that ARF was able to positively regulate the stability of SLUG, a protein that it is able to promote cell migration and cancer metastasis through repression of E-cadherin and the regulation of epithelial-mesenchymal transition. In fact, ARF interacts with and stabilizes SLUG protein inducing its sumoylation. This would result in an increased of prostate cancer progression. Furthermore, by using immunohistochemistry staining, they observed that after inactivation of p19ARF SLUG levels decreased with the concomitant increase of E-Cadherin levels resulting in a reduced rate of prostatic cells proliferation (Xie et al,

2014). Moreover, it has been reported indeed from Humbey and colleagues (2008) that ARF silencing can limit the progression of some tumors such as lymphoma. Primary murine B-cell lymphoma cell lines were infected with a short hairpin directed against ARF transcript and these cells were used for tail vein injection assay in immunocompromised mice. The experiment showed that lymphoma development was impaired in mice injected with ARF knock down cells compared to the controls (Humbey et al, 2008).

These experimental evidences seem to suggest the hypothesis that ARF might have pro-survival functions, implying that it may function in both tumor suppressive and oncogenic pathways in cancers, depending on the cellular context.

In line with this, it has been reported that, depending on cellular context, ARF has also a role in the autophagic process with a pro-survival effect. Autophagy is a lysosome-mediated process of self-digestion that occurs during periods of nutrient deprivation, when proteins and organelles are degraded and the released amino acids are used for the synthesis of essential proteins and ATP to improve cell survival (figure 3) (Humbey et al, 2008). Interestingly, several laboratories have demonstrated a dual role of autophagy in tumor development and progression. In fact, on one hand it has been reported that autophagy is required to block lymphoma and liver cancers progression; on the other hand, there are some data indicating that established tumor cells use autophagy as a crucial survival pathway (Balaburski et al, 2010).

Regarding ARF involvement in the autophagy process it has been reported that, depending on cellular context, ARF expression can have opposite effects on this mechanism. It has been shown from Reef and colleagues (2006) that both mouse and human ARF proteins have a single internal methionine residues (Met45 and Met 48, respectively) used as a start point for a different translational initiation with the generation of a small mitochondrial ARF (smARF). This short form is able to localize to mitochondria although it is well known a nucleous/nucleolar localization of the full-length ARF protein. In

particular, Reef and colleagues observed that after p19ARF overexpression there was a decrease of cell viability, induction of caspase-independent cell death and also increase of the lipidated LC3 (LC3II) levels, a protein commonly used as autophagic marker. In fact, upon induction of autophagy, the predominantly cytoplasmic LC3 protein, LC3I, is conjugated to phosphatidylethanolamine to yield an LC3II form that associates with autophagosomal membranes. Furthermore, Reef and colleagues found an attenuation of cell death induced by smARF overexpression upon Beclin-1 silencing, suggesting a different pro-death function for this short isoform (Reef et al, 2006). Although smARF can activate autophagy, this isoform is very unstable and present at low or undetectable endogenous levels. These data raised the question about the role of full-length ARF protein in autophagy. Recently, it has been demonstrated that both transfected full-length or nucleolar mutant ARF were able to induce cell death and autophagy in HEK 293T cells where p53 is inactive (Abida and Gu, 2008). In addition, Abida and colleagues (2008) reported that in U2OS cells, where the expression of p14ARF was under control of an IPTG-inducible promoter, p14ARF induced autophagy in a p53-dependent manner.

On the other hand, Humbey and colleagues (2008) not only demonstrated that the presence of full length p19ARF is important for lymphoma development but also that, after nutrient deprivation and p19ARF silencing, there was a reduction of LC3II protein levels in p53-null MEFs so indicating that autophagy pathway is impaired. In line with these findings, Humbey and colleagues observed not only the accumulation of p62 protein usually degraded during autophagy but also an impaired cells viability (Humbey et al, 2008). Taken together these data suggest that ARF can have a pro-survival role in autophagy depending on cellular context.

1.4 OTHER FUNCTIONS OF ARF PROTEIN.

Usually, the ARF basal expression levels are low in normal proliferating cells suggesting that in this context ARF have no particular cellular function.

Although it is known that ARF protein levels increase after oncogenic stimuli, it has been reported that basal ARF has a role in mouse eye development. In particular, it has been observed that knockout ARF mice developed normally (Kamijo et al, 1999; Kamijo et al, 1997) despite their eyes were smaller compared to the eyes of wild type mice (McKeller et al, 2002). Moreover, it has been reported that ARF^{-/-} mice display both defects in the neuroretina and the lens which ultimately result in blindness. Upon a closer examination, it has been demonstrated that elements of the hyaloid vascular system (HVS) did not regress in ARF^{-/-} mice after post-natal day 10 compared to wild type mice where HVS normally regress by post-natal day 14. Loss of p53 did not alter the ARF^{-/-} eye phenotype in p53^{-/-} ARF^{-/-} mice. Importantly, these characteristics were not observed in p53^{-/-} mice, indicating that ARF role in hyaloid vascular regression is p53-independent (Ito and Yoshioka, 1999; McKeller et al, 2002).

In addition to ARF expression within the eye, ARF has also a role in male germ cell development in mouse (Gromley et al, 2009; Zindy et al, 2003). In fact, it has been reported that ARF is overexpressed in male spermatogonia, cells that line the basement membrane of each seminiferous tubule. In particular, it has been demonstrated that ARF^{-/-} mice display a reduced sperm number compared to wild type mice with increase of p53-dependent apoptosis during germ cell development (Churchman et al, 2011; Cole et al, 2010).

Furthermore, it has been reported that ARF activity occur also during skin differentiation. Vivo and colleagues (2009) demonstrated that ARF interacts and sumoylates p63 in HaCat cells and in human primary

keratinocytes. In particular, it has been reported p63 decrease and ARF increase after induction of differentiation program in HaCat cells and in human primary keratinocytes (Vivo et al, 2009).

1.5 ARF TURNOVER

When cells are stimulated to proliferate by oncogenic proteins such as Myc, E2F, E1A, oncogenic Ras and v-Abl, ARF levels increase (de Stanchina et al, 1998; DeGregori et al, 1997; Palmero et al, 1998; Zindy et al, 1998). After the exposure to some radiations and genotoxic drugs ARF expression levels increase contributing to the DNA damage response. Moreover, it has been shown that viral infection was able to induce ARF expression resulting in reduction of viral infectivity (Garcia et al, 2006; Sharpless, 2005; Sherr, 2006). Given its strong ability to block growth and proliferation, cells must develop mechanisms that promptly reduce either its expression or functions when its activity is no longer required.

Both mouse and human ARF are relatively stable proteins with an half-life of about 6 hours in tumor cells, and that its turnover is p53 independent (Kuo et al, 2004). In particular, it has been shown that ARF protein is more stable within the nucleolus and that its half life rapidly decrease in the nucleoplasm. This phenomenon could be explained by the fact that in the nucleolus ARF protein can interact with nucleophosmin (NPM/B23) resulting in more stable protein complex formation. Furthermore, it has also been reported that NPM not only can protect ARF from degradation, but also it is important for its nucleolar localization, even though, on the other hand, ARF causes NPM polyubiquitination and degradation (Colombo et al, 2006; den Besten et al, 2005; Itahana et al, 2003). In this way NPM is able to sequester ARF into the nucleolus and to prevent its nucleoplasmic degradation.

Despite the fact that human p14ARF is a lysine-less protein and the murine p19ARF has a single lysine residue not required for proteasome degradation, it has been reported that ARF levels increase after treatment of the cells with proteasome inhibitors. In particular, Pollice and colleagues (2007) experiments have shown an increase of ARF protein levels in 293T cells upon treatment with the proteasome inhibitor MG132 (Pollice et al, 2007). In

general, the fact that ARF levels increase with MG132 treatment suggests that ARF degradation depends, at least in part, by the proteasome even if it is well known that for the vast majority of proteins, conjugation of ubiquitin to internal lysines is the initial event in their degradation by the ubiquitin-proteasome system (Kuo et al, 2004; Pollice et al, 2004; Pollice et al, 2008; Rodway et al, 2004). The proteasome is the site of protein destruction in eukaryotic cells and it is composed of the 20S catalytic associate with the regulatory 19S subunit. Target proteins that need to be degraded are ubiquitinated and then unfolded with consequential translocation into the cavity of the catalytic 20S subunit of the proteasome (Hershko and Ciechanover, 1998). On the other hand, both 20S subunit and 26S proteasome itself are also able to degrade non ubiquitinated proteins even if the mechanism is not yet elucidated (Kalejta et al., 2003; Asher et al., 2005). In fact, it is well known that proteins such as p21, Hif1 α , members of Rb family of tumor suppressors, p53 and p73 can be directed and degraded by the proteasome without ubiquitination. In many cases, if the protein can be delivered to the proteasome in a denaturated or partially unfolded state, ubiquitination should not be required for its degradation (Kong et al, 2006; Pollice et al, 2007; Sdek et al, 2005). Interestingly, it has been reported that ARF can be degraded in vitro by the 20S proteasome in the absence of ubiquitination, and that this process can be counteracted by TBP1, Tat Binding Protein 1, a component of the regulatory subunit of the proteasome (Pollice et al, 2007). In particular, upon ARF-TBP-1 protein interaction, the half-life of ARF protein increased (from 6 to 10 hours) with consequential activation of the MDM2/p53 pathway (Pollice et al, 2004).

Nevertheless, it has been reported by Kuo et al (2004) that, although it lacks Lysine, ARF is subjected to a rare mechanism of ubiquitination that takes place at N-terminal of proteins, independently of p53 and MDM2 (Kuo et al, 2004). All cases of N-terminal ubiquitination have involved mutant proteins from which lysine residues were removed by mutation, and the physiologic

significance of N-terminal ubiquitination has remained unclear. For example, it has been reported that MyoD transcription factor was polyubiquitinated at N-terminus and degraded by the proteasome (Breitschopf et al, 1998). Another case of this rare mechanism it was found to regulate the p21 mutant lysine-less turnover (Sheaff et al, 2000). Recently, Chen and collaborators (2010) identified a specific ubiquitin ligase for ARF called ULF (ubiquitin ligase for ARF) by using a mass spectrometry analysis of the NPM protein complexes. In particular, they observed that ARF and ULF interacted both in vivo and in vitro. Moreover, upon ULF silencing by RNAi, ARF protein levels increase although ARF mRNA levels remained unchanged. In addition, they showed an induction of ARF ubiquitination in a dose-dependent manner upon ULF over-expression. Interestingly, they found that proteasome-mediated ARF degradation is inhibited in cancer but not in normal cell lines where ARF half life is reduced to only 30 minutes. Interestingly, they observed that oncogenic stress such as c-Myc expression abrogates ULF-mediated ARF ubiquitination by interaction of c-Myc with ULF; this interaction promotes ARF-dependent, p53- mediated growth arrest (Chen et al, 2010).

It is also worth mentioning that it has been very recently reported that MDM2 intracellular increase can, indeed, cause ARF destruction by the proteasome, leading to the hypothesis that in an oncogenic environment in which MDM2 is overexpressed, ARF levels are kept low due to a rapid turnover caused by MDM2 triggered degradation (Vivo et al 2015).

Finally, it has been reported that also the Protein Kinase C (PKC) is involved in the regulation of ARF stability. Accumulating data suggest that various PKC isoforms participate in the regulation of cell proliferation, differentiation, survival and death (Nishizuka, 1995). Intracellular and tissue distribution together with protein phosphorylation give substrate selectivity to these kinases so ensuring a fine tuning of PKC activation. The positive correlation between tumor promoting effects of various phorbol esters with their ability to activate certain PKC isoforms indicated that PKC activation is a

critical step in skin tumor promotion.

In particular, in a paper published in 2005 from Inoue and Shiraishi it was well shown that the α PKC isoform (PKC α) is involved in the stabilization of exogenously expressed ARF protein level. In fact, they found that after activation of PKC by TPA exogenously expressed ARF protein levels were stabilized. In addition, it has been shown that TPA treatment was able to stabilize p14 ARF protein in cells expressing a temperature sensitive mutant of p53 suggesting that TPA-mediated stabilization of p14ARF protein is p53-independent (Inoue and Shiraishi, 2005).

2. PRELIMINARY DATA AND AIM OF THE STUDY.

In the last years several laboratories, including our, started to investigate the mechanisms regulating ARF protein turnover. When I joined the Prof. La Mantia lab, dott. M. Vivo had already started to analyze the role of Protein Kinase C in the ARF stability regulation.

Different experiments have revealed an increase of both p14ARF (transcript and protein levels) and PKC upon calcium induced keratinocyte differentiation (Vivo et al., 2009; Inoue et al., 2005). In addition, *in silico*-examination of p14ARF protein sequence revealed three potential target sites for phosphorylation mediated by PKC. Among the potential PKC target sites Threonine at position 8 lies within the most conserved region (amino acids 2-14) required for the activity and nucleolar localization of ARF protein. Preliminary data also showed that ARF wild type was phosphorylated *in vitro* by PKC catalytic subunit. Furthermore, it was demonstrated that TPA-induced PKC activation results in the stabilization of cytoplasmic ARF. Moreover, in various cell lines, p14ARF interacts with PKC (Vivo et al., 2013).

On the basis of these data, the aim of my study initially was to analyze the role of phosphorylation on the biological activity, cellular localization and stability of ARF protein (Vivo et al., 2013). In addition, our findings on ARF stabilization in the cytosol prompted us to investigate whether the presence of ARF in the cytoplasm could result in partially unknown functions. Therefore, we decided to examine the role of endogenously expressed ARF protein in different cell lines upon its silencing by RNA interference (manuscript in preparation).

3. MATERIALS AND METHODS

Cell cultures, Cloning, Protein expression and reagents.

The human epithelial cell line HaCaT was obtained from Dr. Antonio Costanzo, Department of Dermatology, University of Rome Tor Vergata, 00133 Rome, Italy. All other cell lines (U2OS, H1299, Hela and MCF-7) were purchased from the American Type Culture Collection (ATTC). Cells were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum (Euroclone, Life Science) at 37 °C in a humidified atmosphere of 5% (v/v) CO₂ in air.

For DNA transfections the cells were transfected using LipofectAMINE 2000 reagent (Invitrogen). For siRNA experiments the cells were transfected with RNAi Max reagent (Invitrogen).

Antibodies used in this study: anti PARP-1 antibody (Beta HSer660) (from Cell Signaling Technologies 9542, Boston, MA, USA), anti ARF C-18, anti actin I-19, anti p53 DO-1, anti MDM2 SMP-14, (from Santa Cruz); anti ARF Ab2 14PO2 (Neomarkers); 6XHIS monoclonal antibody (Clontech); anti myc antibody 06549 (Upstate); anti X-press monoclonal antibody (Invitrogen); anti pThreonine Q7 (Qiagen); anti-DAPK 5S clone (Sigma).

For cloning of ARF mutants and Western blot (WB) analysis see below the attached paper.

Phosphorylation and co-immunoprecipitation assays.

Immunoprecipitation assay was performed using 4 mg of total protein extracts of H1299 treated or not with TPA. The complexes were incubated with anti ARF antibody and then, analyzed with SDS-PAGE elettrophoresis followed by blotting with anti p-Threonin antibody.

Co-immunoprecipitation assay was performed using 1 mg of total protein extracts of U2OS cell line. In particular, the cells were transfected with

expression plasmids encoding human MDM2 or Myc tagged p32, and/or Xpress tagged wild type or mutant ARF proteins. Protein extracts were immunoprecipitated with anti Xpress or anti-ARF antibodies. The complexes were blotted and probed with anti-MDM2, anti-Myc and anti ARF antibodies. For all the informations about buffers used in these experiments see below the attached paper.

Subcellular localization.

For subcellular localization analysis U2OS cells were plated on micro cover glasses and then, transfected with expressing plasmid encoding 6XHis tagged wild type and mutant ARF. After 24 hours, the cells were fixed, permeabilized and incubated with primary monoclonal antibody against 6XHis tag and, then, with anti-mouse as secondary antibody. 100-150 cells were analyzed under a fluorescence microscope and the counts plotted in a graph. For informations about statistical analysis see the attached paper.

Protein turn over analysis.

For protein turnover analysis U2OS cells were plated and, after 24 hours, treated or not with protein synthesis inhibitor Cycloeximide for 0, 4, 6, 8 and 10 hours. Total protein extracts were analyzed with western blot using anti-ARF and anti-actin antibodies. For information about the concentration of inhibitor and antibody used, see the attached paper.

Proliferation analysis.

For proliferation analysis U2OS cells were plated in a six well multiplates and transfected with plasmids encoding wild type and mutant ARF proteins. The cells were treated with G418 for 10 days and then, fixed and stained with crystal violet. After 20 minutes, the cells were decolorated and the wash solution was used to measure the absorbance.

P53 stabilization assay.

For p53 stabilization assay U2OS cell were transfected with increased amount of plasmids encoding wild type and mutant ARF proteins. Total protein extracts were analyzed with western blot using anti-ARF, anti p53 and anti-actin antibodies.

For all the informations about plasmid concentrations and antibodies see below the attached paper.

siRNA of ARF.

A duplex siRNA oligomer designed to target ARF was obtained by INVITROGEN

The siRNA sequence for ARF used is :

- siRNA sens 1 : GAAGAUCAAGGUCAUGAUGA dTdT.
- siRNA antisense 1 : UCAUCAUGACCUGAUCUUC dTdT

The cells were transfected with siRNA (INVITROGEN) composed by the same nucleotides of siARF but in random arrangement (siSCR) as negative control. In addition, a siRNA targeting a sequence firefly luciferase mRNA (Quiagen) was used as other negative control. Hela or H1299 or MCF-7 cells were transfected with siRNAs (final concentration 50µM) and left for 48 or 72 hours. Western blots were performed with antibodies directed against ARF, PARP-1, actin or GAPDH as loading control.

siRNA of DAPK.

A duplex siRNA oligomer designed to target DAPK (Quiagen) was purchased from Qiagen and used at the final concentration of 50µM. Hela cells were silenced for 72 hours and then, analyzed by western blot with antibodies directed against ARF, PARP-1, DAPK and actin as loading control.

Treatment with apoptosis inhibitor Z-VAD-fmk.

After 48 hours of silencing, ARF depleted Hela cells were

trypsinized and replated in presence of Z-VAD-fmk (BioVision) for 5 hours (10 mM stock). Z-VAD-fmk is a synthetic peptide that irreversibly inhibits activity of caspase family protease and block apoptosis. Western blot was performed with antibodies directed against ARF C-18, PARP-1 and actin as loading control.

Light microscopy.

Hela or H1299 cells were silenced with siRNA directed against ARF and negative controls (siLUC or siSCR) for 48 or 72 hours. Then, the cells were trypsinized and replated. The cell shape was analyzed using light microscopy at 5 or 8 hours post-replating. The images of light microscopy were obtained using the Nikon eclipse TE2000-U microscope at 20X magnification. The percentige of cells with round phenotype was reported in a graph that represent mean values \pm SD of 3 independent experiments.

4. RESULTS

4.1 Mutation of Threonine 8 does not affect ARF folding.

ARF protein sequence has different potential target sites for phosphorylation such as Threonine (T8, T31, T73, T95) and Serine (S52, S64, S92, S127) residues. In particular, *in silico*-examination of p14ARF protein sequence revealed three potential target sites for phosphorylation mediated by PKC. Among the potential target sites for PKC mediated phosphorylation of ARF protein, threonine at position 8 lies within the most conserved region (amino acids 2-4) required for ARF activity and nucleolar localization (Figure 4). To understand the role of this residue in ARF function, in our laboratory were constructed ARF mutants where threonine 8 was replaced with an alanine or an aspartic acid residues, generating mutants that cannot be phosphorylated (T8A mutant) or that mimics the phosphorylated status of the protein (T8D mutant), respectively.

In order to verify whether the substitution of threonine 8 could have resulted in an incorrectly folded protein, we decided to test the ability of p14ARF mutants (T8A and T8D) to bind two well known ARF partners, such as MDM2 and p32, interacting with the ARF N-terminal and C-terminal domains respectively (Itahana and Zhang, 2008; Weber et al, 2000).

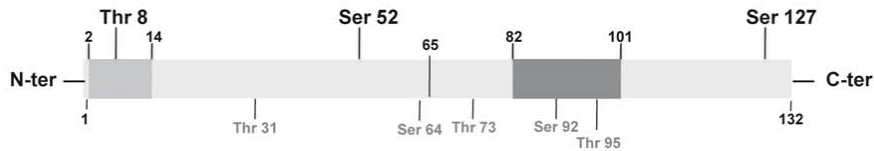
To this purpose we performed experiments of co-immunoprecipitation in U2OS cell lines which express undetectable levels of p14ARF. We transfected U2OS cells with expression plasmids encoding human MDM2 in combination or not with X-press tagged wild type or mutant ARF. Total protein extracts were subjected to immunoprecipitation with antibody directed against the X-press tag. Immunoprecipitated complexes were analyzed by using SDS-PAGE electrophoresis followed by immunoblot with antibodies directed against MDM2 and p14ARF. As shown in the input panel in figure 5 (a) (on the left side), ARF and MDM2 were efficiently expressed only when transfected. In addition, as reported in the immunoprecipitation panel (on the right in the

figure), we were able to see the bands corresponding to both wild type and mutants ARF and MDM2 only where cotransfected. These data indicate that the substitution of Threonin at position 8 does not affect ARF binding to MDM2.

Then, to verify the correct binding of ARF mutants to p32, we decided to transfect U20S cells with expression plasmids encoding Myc tagged p32 with wild type or mutants ARF. Immunoprecipitated complexes were blotted and probed with anti Myc and p14ARF. In the input in figure 5 (b) we were able to see the bands corresponding to p32 and ARF only were transfected. In addition, we obtained a co-immunoprecipitation of both wild type and mutant ARF and p32 proteins where co-transfected. Also in this case, the experiment showed that T8A and T8D mutant proteins were able to bind p32 as wild type ARF protein.

Taken together these data indicate that both substitutions of threonin 8 do not affect ARF binding properties, so suggesting that folding of the protein is not compromised by the mutations.

p14ARF phosphorylation sites predicted by GPS 2.1 software

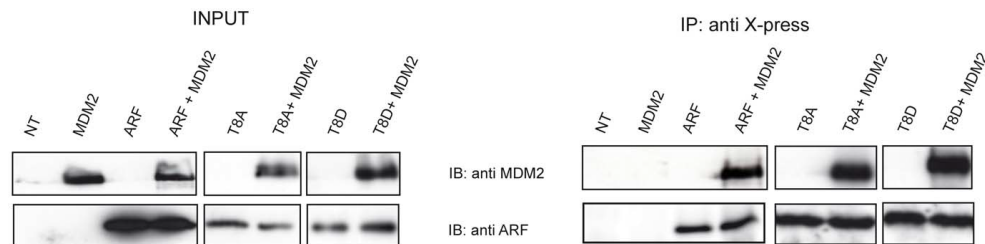


Vivo et al, 2013.

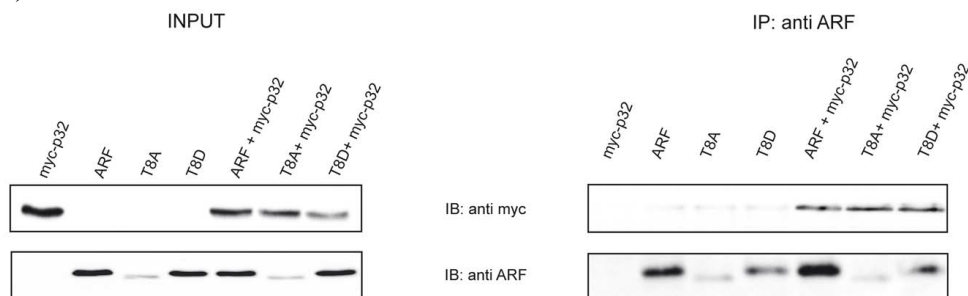
Figure 4. Scheme of predicted p14ARF phosphorylation sites.

Diagrammatic scheme of p14ARF phosphorylation sites identified in silico. In light gray is indicated the conserved 2-14 amino acid stretch within the N-terminal exon 1 β encoded domain (amino acid 1-65), while in dark gray the exon 2 nuclear localization signal. PKC phosphorylation sites are shown in bold above the scheme, while other potential kinases target sites are denoted in gray below the scheme (Vivo et al, 2013).

A)



B)



Vivo et al, 2013.

Figure 5. Mutation of Threonine 8 does not affect ARF binding to MDM2 and p32.

Co-immunoprecipitation experiments are shown in both A) and B) panels.

A) X-press tagged plasmids encoding ARF mutants or wild type proteins were transfected in U2OS cells in combination or not with plasmid encoding human MDM2 protein. Total protein extracts were subjected to immunoprecipitation with antibody directed against the X-press tag and analyzed using western blot analysis with anti MDM2 and p14ARF antibodies. B) U2OS cells were transfected with plasmid encoding myc tagged p32 in combination or not with wild type and mutants ARF. Protein extracts were immunoprecipitated with anti ARF antibody followed by immunoblot with anti Myc and ARF antibodies (Vivo et al, 2013).

4.2 Mutation of Threonine 8 affects ARF protein turn-over.

Western blot analysis repeatedly showed that the expression levels of T8A mutant was lower compared to T8D mutant and wild type proteins. We thus decided to evaluate if the substitution in Threonine 8 could affect the stability of ARF protein.

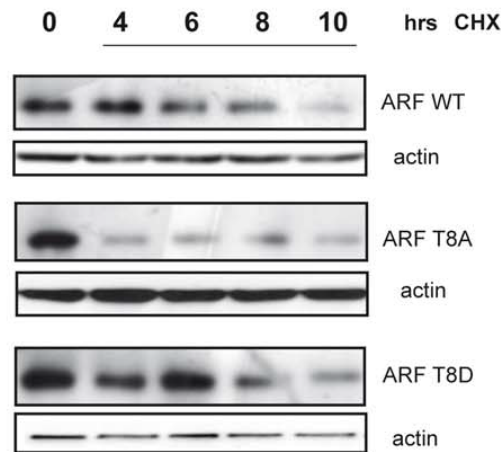
To this aim, we performed an experiment to measure the half-life of wild type and mutant ARF proteins.

U2OS cells were transfected with expression plasmids encoding ARF wild type or mutants. After 24 hours the cells were treated with cycloheximide and collected at different time points (0, 4, 6, 8, 10 hours). Total protein extracts were blotted and probed with anti ARF and anti ACTIN (as internal control).

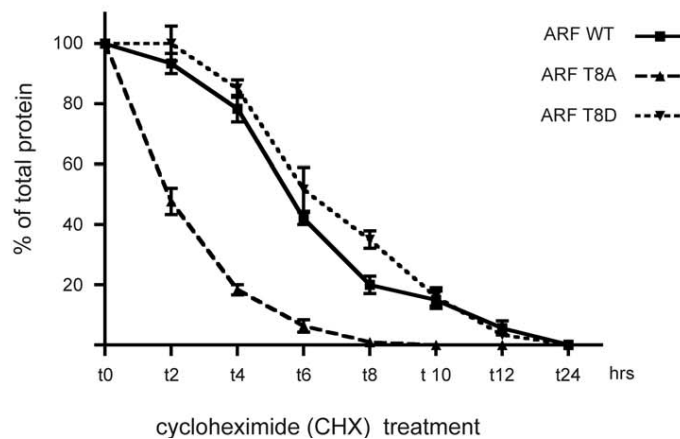
As shown in figure 6 A, the half-life of wild type protein is about 6 hours as reported previously (Pollice et al, 2004). T8D mutant showed an half-life comparable to wild type protein. In line with its reduced level of expression, T8A mutant showed an half-life of about 2 hours after cycloheximide treatment. Western blot data were quantified by normalizing band intensities with actin protein levels. As shown in the graph reported in figure 6 B, while T8D mutant stability is comparable to that of wild type, the half life of T8A mutant is strongly reduced.

Taken together, these data indicate that substitution of Threonine 8 affects the half-life of ARF protein and, therefore, that this residue can be involved in the regulation of p14ARF stability.

A)



B)



Vivo et al, 2013.

Figure 6. Substitution of Threonine 8 affects p14ARF stability.

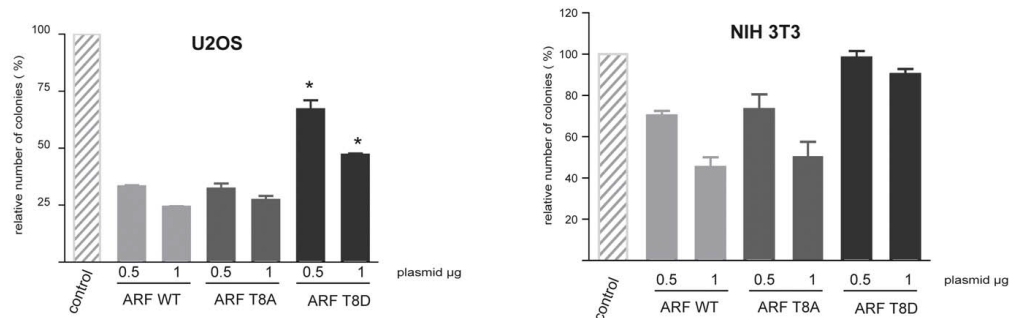
A) U2OS cells were transfected with equal amounts of p14ARF wt, T8A and T8D encoding plasmids and after two days treated with cycloheximide (CHX) for 4, 6, 8, 10 hours. Total protein extracts were analyzed by SDS-PAGE followed by immunoblotting with anti ARF and ACTIN antibodies. B) The plot represents half life analysis of wt and mutant ARFs. Each profile represents the mean of three independent transfections and WB experiments. Band intensities were quantified by Image J analysis and actin normalized before being plotted in graph. The amount of protein at different time points is expressed as percentage of total protein, i.e. protein amount at t0. Standard deviations are also shown (Vivo et al, 2013).

4.3 Mimicking ARF phosphorylation inhibits ARF biological activity.

Next, we decided to test ARF mutants ability to block cell proliferation as wild type protein. We performed Colony Formation Efficiency assay (CFE assay) in U2OS and NIH 3T3 cells where p53 pathway is intact. Cells were transfected with expression plasmids encoding ARF wild type and mutants and then treated with G418 selection for ten days. Subsequently, the cells were fixed and coloured by cristal violet and then the colonies counted. As reported in figure 7, the experiment showed that T8A mutant, that cannot be phosphorylated, was able to block cell proliferation with similar efficiency of wild type protein. On the other hand, ectopic expression of T8D mutant that mimics the phosphorylated status of the protein was instead, less efficient.

Next, based on the evidences that ARF overexpression can induce cell cycle arrest and/or apoptosis in a p53-dependent manner (Calabro et al, 1999; Hashemi et al, 2002; Pollice et al, 2004), we decided to verify whether substitution at threonine 8 could impair ARF ability to activate p53. U2OS cells were transfected with increasing amount of expression plasmids encoding wild type or mutant ARF proteins. Total protein extracts were subjected to SDS PAGE elettrophoresis followed by immunoblot with an antibody directed against p53, ARF and ACTIN (as internal control). As shown in figure 8 both T8A and T8D were able to stabilize p53 with similar efficiency of wild type protein. These data indicate that substitution of threonin 8 does not affects ARF mediated p53 stabilization although both of these mutants showed lower expression levels compare to wild type protein.

COLONY FORMATION ASSAY



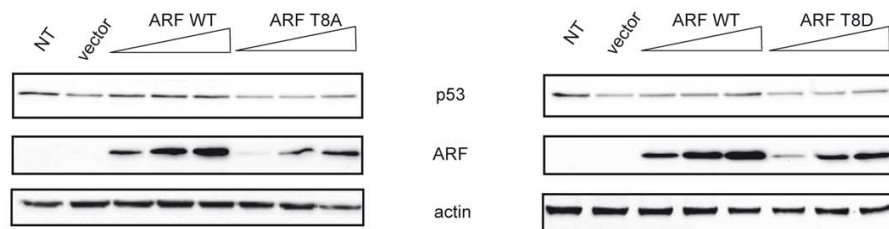
Vivo et al, 2013.

Figure 7. Growth suppression by wt and mutant ARF proteins.

Both graphs indicate ARF and mutants ability to induce growth suppression using a Colony Formation Efficiency (CFE) assay. U2OS and NIH cells were transfected with the indicated amounts of ARF and mutant expression vectors. After selection with G418, colonies were fixed, stained with crystal violet and counted. The plot represents the percentage of colonies obtained with the indicated plasmids relative to that detected with the empty vector. Values represent the mean of four independent experiments. Standard deviation for each value is also given. Asterisks (*) indicate statistically significant differences ($p < 0.001$) for the mutant samples compared to wild type ARF (Vivo et al, 2013).

p53 stabilization

U2OS



Vivo et al, 2013.

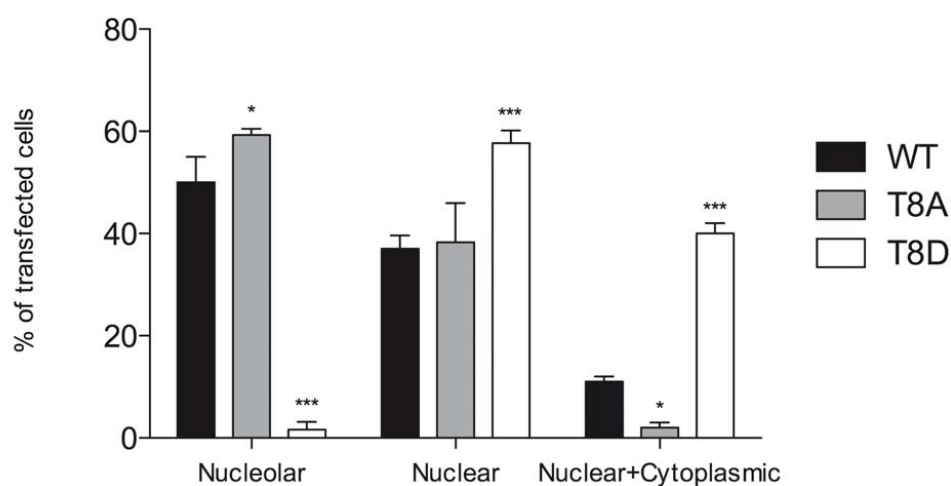
Figure 8. ARF wild type and mutants mediate p53 stabilization.

Western blot showing p53 stabilization following ARF (wt and mutant) transfection in U2OS cells. U2OS cells were transfected with increased amount of plasmids encoding ARF wild type and mutants. Protein extracts were analyzed by western blot with anti p53, ARF and ACTIN antibodies (Vivo et al, 2013).

4.4 Mimicking Threonine 8 phosphorylation induces ARF accumulation in the cytoplasm and nucleus.

It has been extensively shown that ARF amino acid stretch 2-14 is not only involved in MDM2 binding and ARF mediated cell cycle arrest but also contribute to p14ARF nucleolar compartmentalization (Weber et al, 2000; Pollice et al, 2004). On the basis of this informations, we decided to analyze the effect of Threonine 8 substitution on ARF cellular localization. Thus, we analysed the effect of T8 mutations on ARF subcellular localization following transfections in U2OS cells.

U2OS cells were transfected with plasmids encoding his tagged wild type or mutant ARF and then analized by immunofluorescence assay with anti histidine antibody. We counted the number of transfected cells showing nuclear, nucleolar and cytoplasmic localization and we reported in a graph the percentage of the cells with each distribution pattern. As reported in figure 9, 90% of cells transfected with wild type ARF showed a nuclear/nucleolar localization and only 10% of them a nuclear/cytoplasmic distribution. Substitution of threonine 8 with alanine residue did not altered the percentage of cells with nuclear/nucleolar localization. In contrast, T8D mutant, where threonine 8 was replaced with aspartic acid, showed a consistent increase in the percentage of cells with nuclear/cytoplasmic localization.



Vivo et al, 2013.

Figure 9. Mutations of Threonine 8 change ARF localization.

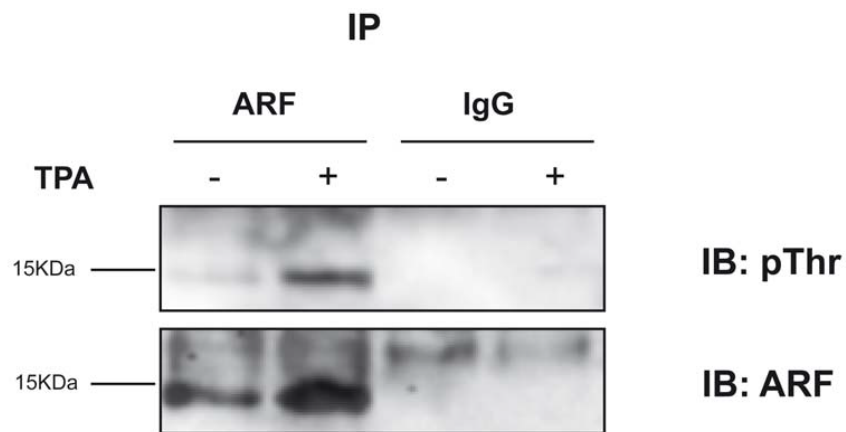
U2OS cells were transfected with p14ARF expression plasmid (wt or mutants) and visualized by IF with anti histidine antibody. Images were taken with a Nikon fluorescent microscope. The histogram, representing the mean of three independent experiments, reports the percentage of transfected cells showing each localization pattern. Standard deviations are also shown. Asterisks indicate statistically significant differences between the sample and wt ARF (* = $p < 0.05$; *** = $p < 0.001$) (Vivo et al, 2013).

4.5 Cytoplasmic localized ARF protein is phosphorylated.

Experiments of nuclear-cytoplasmic fractionation performed by Dott.ssa Maria Vivo revealed that, following treatment with PKC activator (TPA), the cytoplasmic pool of ARF increased. In addition, after lambda phosphatase treatment of cytoplasmic extracts, an increase of ARF band intensity was specifically observed in treated samples. These data suggested that, after phosphatase treatment, multiple phosphorylated ARF species were present in the cytoplasm and converged to the 15Kd band.

We, then, decided to verify whether p14ARF protein could be phosphorylated in cell culture. H1299 cells expressing high levels of p14ARF protein, were treated with TPA for 2 minutes. Total protein extracts were subjected to immunoprecipitation with anti ARF antibody and with IgG as negative control. Immunoprecipitated complexes were analyzed by SDS PAGE electrophoresis and blotted with an antibody directed against phosphorylated threonine (pThr antibody). The experiment shows a 15 KDa band in untreated sample whose intensity increases following TPA stimulation (Figure 10 upper panel). Identity of this band was confirmed by western blot with anti ARF antibodies (Figure 10 lower panel).

Taken together these data confirm that ARF is phosphorylated not only in vitro but also in cell culture.



Vivo et al, 2013.

Figure 10. ARF is phosphorylated in cell culture.

ARF is phosphorylated in H1299 cell line. H1299 protein extracts were immunoprecipitated with anti ARF antibody and, then, analyzed by western blot analysis using anti p-Threonine antibody (upper panel). In the lower panel is shown the efficiency of the immunoprecipitation obtained after stripping and incubation of the membrane with anti ARF antibody (Vivo et al, 2013).

4.6 ARF loss blocks cell proliferation in both Hela and HaCat cells.

Our data revealed that a substitution of Threonine 8 mimicking a phosphorylated status of ARF protein influences its activity and cellular localization. In particular, T8D mutant shows a different cellular localization pattern with increased nuclear/cytoplasmic distribution. In addition our data seems to point to a p53 independent function of ARF specifically impaired by the phosphorylation.

Our experiments led to the hypothesis that in a tumor environment, where PKC is activated, ARF levels increase but its growth inhibitory function might be at the same time impaired. Our observations might thus uncover a novel mechanism through which cancer cells could escape ARF surveillance during cancer progression. Nevertheless, we could suppose that the presence of ARF in the cytoplasm could results in partially unknown functions. Therefore, we decided to investigate ARF role in several cancer cell lines.

To this aim, we performed RNA interfering experiments by using stealth siRNA specifically targeting ARF exon 1 beta to have a long lasting depletion of our protein. We used Hela cells expressing endogenously ARF protein where although not irreversibly, the p53 pathway is disabled due to enhanced protein degradation mediated by the viral protein E6 (Johnson et al, 2002; Wesierska-Gadek et al, 2002). In line with this, ARF overexpression in these cells has no effect on cellular proliferation.

Hela cells were transfected with siRNA directed against either ARF transcript or Luciferase as a negative control. After 6 days, silenced cells were fixed and stained with crystal violet and then, the absorbance of the wash used to decolorate the cells was misured. As shown in figure 11, in ARF silenced cells viability consistently decreased as compared to control siLUCIFERASE (siLUC) cells, suggesting that the ARF expression is required to guarantee cell proliferation.

To verify if this effect was cell type specific, we performed the same experiment in H1299 lung cancer cells (p14ARF +/+), in breast cancer cells

MCF-7 (p14ARF null) as negative control (figure 12) and in immortalized HaCat cells where ARF is almost exclusively localized in the cytoplasm (data not show).

Only in HaCat cells ARF loss resulted in decreased viability. These data suggest that ARF is not required to guarantee cell proliferation in all the cellular contexts.

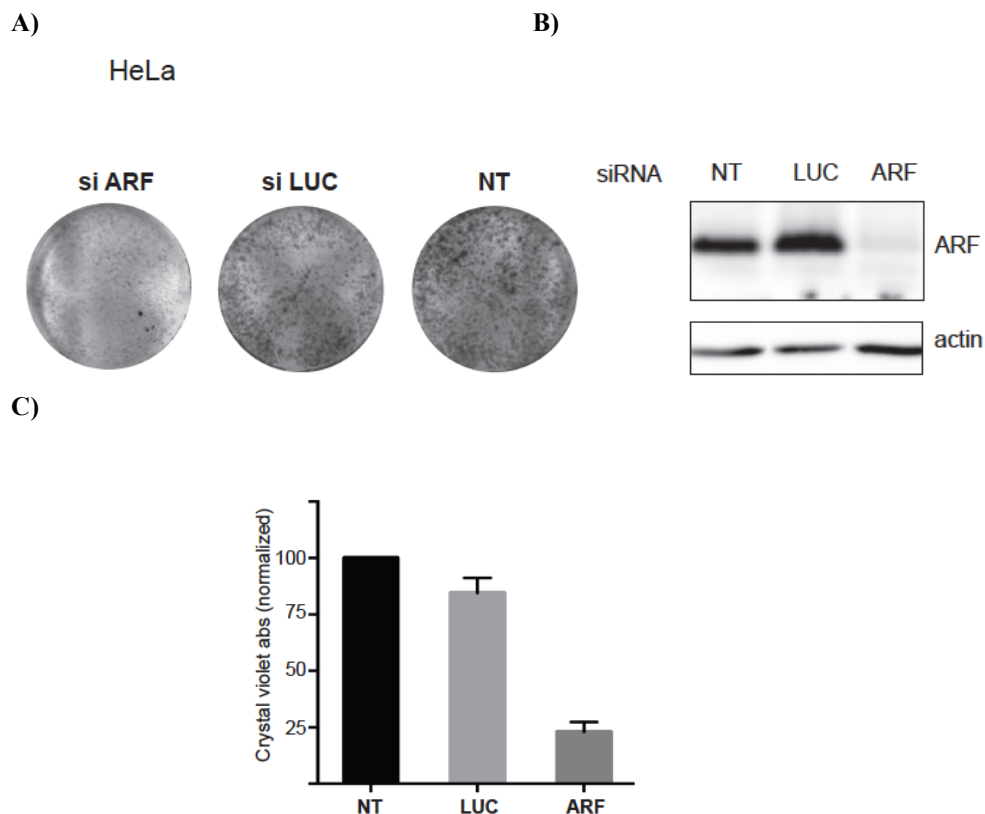


Figure 12. Growth suppression after ARF silencing in HeLa cells.

A) HeLa cells were transiently transfected with the indicated siRNAs for 5 days, and then cell viability was analysed by crystal violet staining (CVS assay). B) Silencing efficiency was analysed by western blot with anti ARF and anti actin as loading control. C) The absorbance of each well (NT, siSCR and siARF) was calculated as percentage of the control wells (NT) set to 100%. Graphs represent mean values \pm SEM of 3 independent experiments.

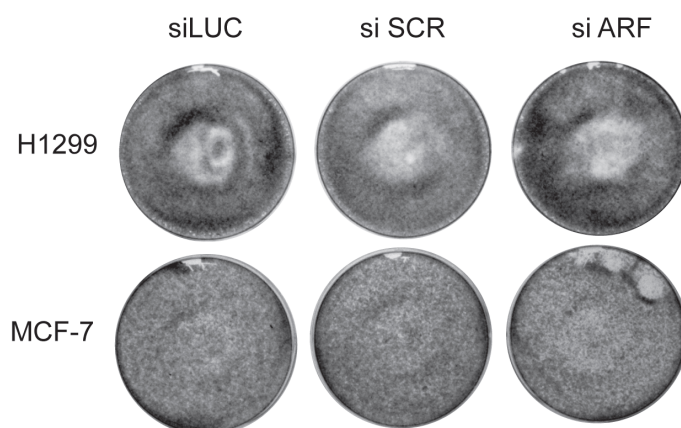


Figure 12. ARF silencing cells does not affect cell growth in H1299 and MCF-7 cells.

Cells were transfected with the indicated siRNA and incubated for 6 days and, then, cell viability was analysed by CVS assay as described.

4.7 ARF loss determines a round phenotype of the cells.

During our silencing experiments we observed that depleted cells were smaller than controls and acquired round phenotype. To get further inside this phenomenon, Hela cells were transfected with siRNA directed against ARF, or luciferase, or with a siRNA composed by the same nucleotides of siARF but in random arrangement (siSCR). After 72 hours of silencing and to follow the adhesion process, Hela cells were detached from the plate by trypsinization, replated and analyzed by both western blot and light microscopy at 5 and 24 hours post-plating. As shown in figure 13 A, 5 hours after replating, control cells were almost completely spread. On the other hand, ARF depleted cells although adherent to the substrate, showed a round phenotype that become more evident at 24 hours post plating when the control cells have completed adhesion and spreading processes.

In parallel, as reported in figure 13 B, we collected cellular protein extracts for a western blot analysis and, in line with the previous data, we observed that both 5 and 24 hours after replating, ARF depleted cells showed PARP-1 cleavage indicating the commitment of apoptotic process. At this point, we also performed ARF silencing in H1299 cells where we previously observed that ARF loss did not reduced cell viability (Figure 14). In particular, as shown in the western blot analysis (figure 14 A), we did not observe a PARP-1 cleavage both 48 and 72 hours after silencing and 8 hours after replating. Nevertheless, light microscopy images reported in figure 14 B showed the presence of cells with a round phenotype in siARF treated cells compared to the controls.

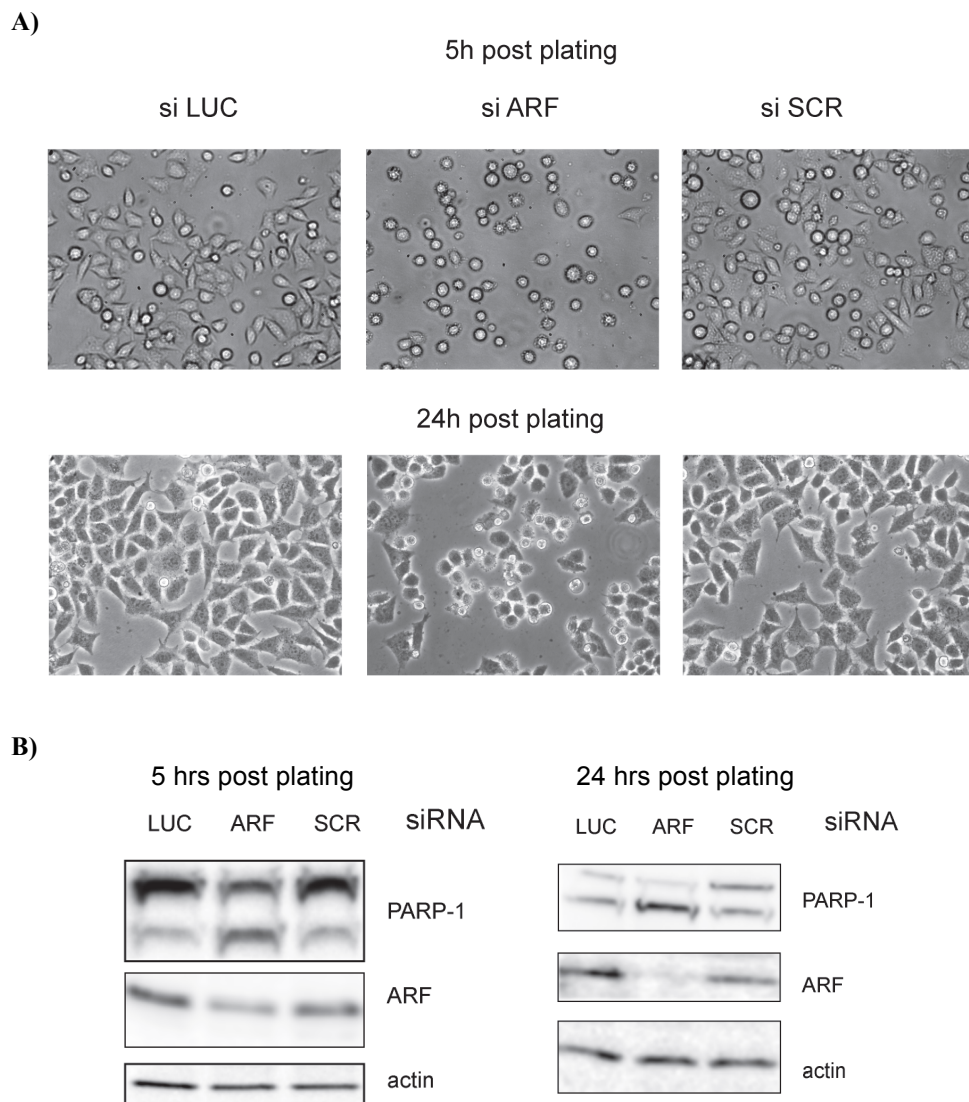
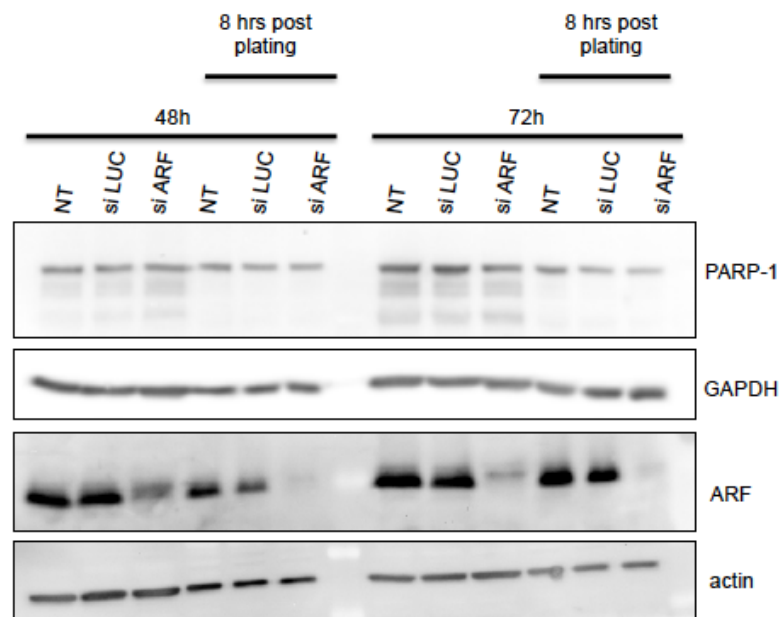


Figure 13. p14ARF loss influences cell morphology and apoptosis.

HeLa cells transiently transfected with the indicated siRNAs for 48h, were detached by trypsinization and replated at a density of 1×10^5 /ml in six-well plates. Images were collected by phase-contrast microscope 5 and 24h post plating (A). Silencing efficiency and apoptosis were analysed by western blot with anti ARF, anti PARP-1 and anti actin as loading control (B).

A)



B)

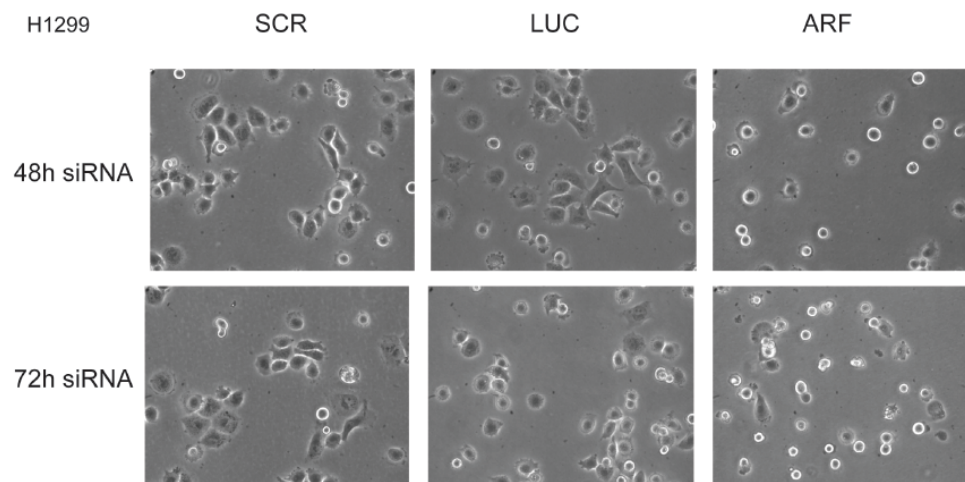


Figure 14. ARF silencing determines round phenotype but not PARP-1 cleavage in H1299 cells.

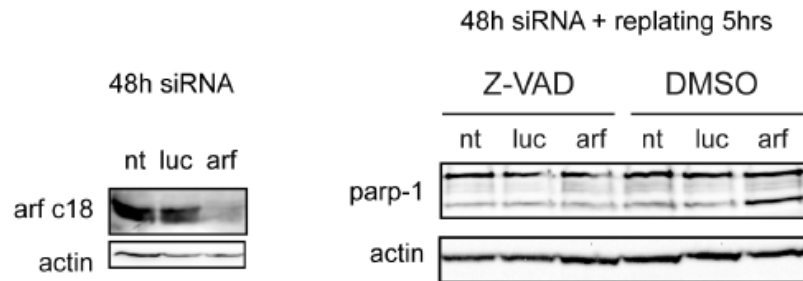
H1299 cells were transfected with the indicated siRNA. After 48 and 72 hours from silencing, the cells were detached by trypsinization and replated at a density of 1×10^5 /ml in six-well plates. Cells were analyzed by western blot with anti ARF and PARP-1 antibodies and actin as loading control (A). Images were collected by phase-contrast microscope 8 hours post plating (B).

4.8 Round phenotype is not apoptosis-dependent.

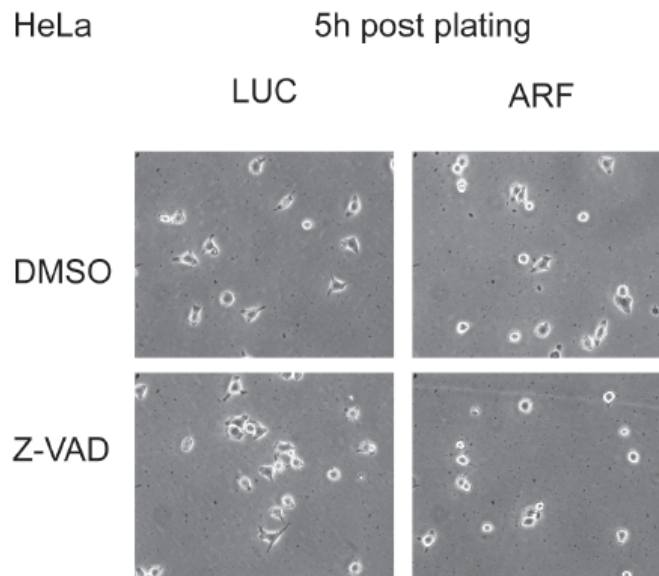
On the basis of the fact that ARF silenced Hela cells showed not only a round phenotype but also started the apoptosis process, we wondered whether these two phenomena were connected or not. To this purpose, Hela cells were transfected with siRNA directed against ARF or Luciferase (as negative control). 48 hours after silencing, the cells were trypsinized and replated in presence of Z-VAD-fmk, which is an apoptosis inhibitor, for 5 hours. Western blot, reported in figure 15 A, showed that following Z-VAD treatment PARP-1 cleavage was inhibited in these experimental conditions. Next, we analyzed cell shape by light microscopy and cells treated with Z-VAD, where apoptotic program was inhibited, still showed the round phenotype (Figure 15 B). We reported these results in a graph where we compared the number of cells with round phenotype in control and siARF cells (Figure 15 C).

Both these data and the results obtained in H1299 suggest that the round morphology of ARF silenced cells was not a consequence of apoptotic program engagement.

A)



B)



C)

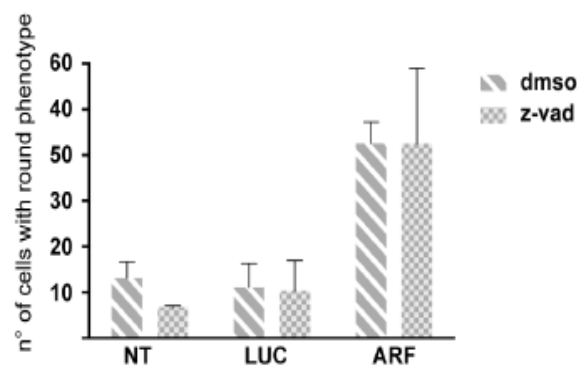


Figure 15. ARF silenced cells show round phenotype after inhibition of apoptosis mediated by Z-VAD treatment.

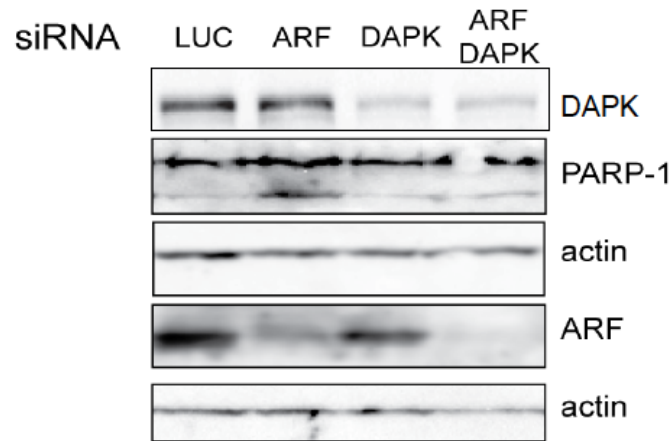
Hela cells were transfected with siARF and siLUC and, after 48 hours, trypsinized and replated as described. Cells were treated with Z-VAD for 5 hours and, then, the cell shape analyze by light microscopy (A) and the number of round cells reported in a graph (B). Graphs represent mean values \pm SD of 3 independent experiments. Total protein extracts were analyzed by western blot with anti ARF and PARP-1 antibodies (C).

4.9 ARF loss induces DAP Kinase-dependent apoptosis.

Experiments performed by Dott.ssa Maria Vivo in HeLa cells grown in monolayer or in suspension have revealed that, after ARF silencing, only the cells in monolayer showed a PARP-1 cleavage suggesting that the establishment of cell-matrix engagement is required to induce apoptosis. It has been reported that Death Associated Protein Kinase (DAPK) exerts apoptotic effects by suppressing integrin functions and integrin-mediated survival signals, thereby activating a p53-dependent apoptotic pathway (Wang et al, 2002). To determine if the observed apoptosis was DAPK dependent, we performed ARF depletion experiments in presence or absence of DAP kinase expression. Cells were treated with ARF siRNA alone or together with DAP kinase specific siRNA in order to simultaneously abrogate both proteins expression. Total protein extracts were subjected to SDS-PAGE and blotted with anti PARP-1, ARF, DAPK and ACTIN (as loading control). As shown in figure 16 A, we observed that PARP-1 cleavage and apoptosis were not detected upon simultaneous ARF and DAPK silencing. Taken together these data suggest that ARF loss induces DAP Kinase-dependent apoptosis. In line with our previous results ARF/DAPK silenced cells show a round phenotype, confirming that this cell morphology is not connected with the induction of apoptosis program (figure 16 B).

It has been reported that DAPK can induce apoptosis via p53 activation (Pei et al, 2014). Thus, we decided to verify p53 status after ARF silencing. HeLa cells were silenced for 72 hours and then the protein extracts analyzed by western blot. As shown in figure 17, we observed an increase of p53 total levels in ARF silenced cells suggesting that the DAPK induced apoptosis could be p53-dependent.

A)



B)

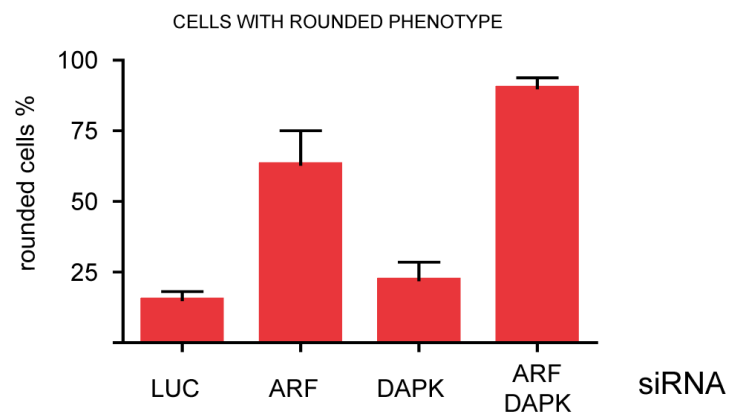


Figure 16. DAPK response is required for the induction of apoptosis but not for the acquisition of round phenotype in ARF silenced cells.

HeLa cells were transfected with siARF or siDAPK alone or in combination. After 72 hours, protein extracts were analyzed by western blot using anti ARF, PARP-1, DAPK and ACTIN antibodies (A) and cells shape was analyzed by light microscopy as described (B). Graphs represent mean values \pm SD of 3 independent experiments.

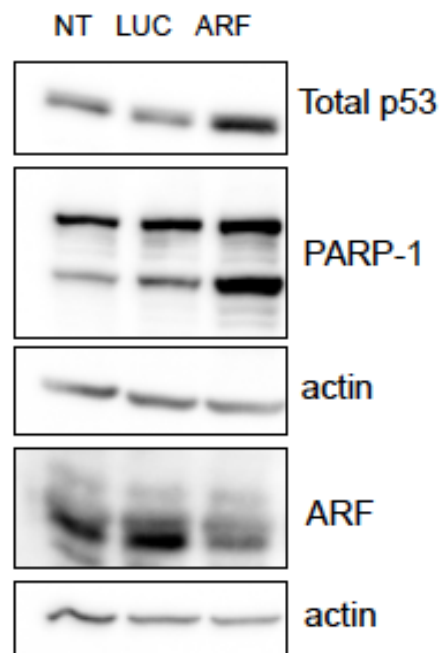


Figure 17. Effect of ARF silencing on p53 levels.

Hela cells were transfected with the indicated siRNA and, after 72 hours, protein extracts were analyzed by western blot with anti ARF, p53 and PARP-1 antibodies. Actin is a loading control.

5. DISCUSSION.

Mammalian cells that sustain oncogenic insults, after a discrimination between physiological and noxious growth promoting states, can invoke defensive programmes resulting in a cell cycle arrest and/or apoptosis (Sherr, 2006).

In normal conditions ARF protein is kept at lower levels but, when the cells are stimulated to proliferate by oncogenic stimuli, ARF levels increase resulting in cell-cycle arrest or apoptosis in a p53-dependent or independent manner. (Eymin et al, 2003; Ozenne et al, 2010; Weber et al, 2000).

Despite its role in growth suppression, ARF is overexpressed in a significant fraction of human tumors. Moreover, it has been reported that its silencing can limit the progression of some tumors suggesting a possible pro-survival ARF function, depending on the cellular context (Chen et al, 2009; Humbey et al, 2008; Xie et al, 2014).

On the other hand, ARF can interact with a cohort of different partners and become a part of different cellular processes making difficult the formulation of a unique ARF role in the cells.

In the past years, intensive studies have been focused not only on ARF activation at transcriptional level but also on the mechanisms regulating ARF protein turnover. In fact, it has been demonstrated that ARF degradation can be regulated by the proteasome by both ubiquitin independent or lysine-independent N-terminal ubiquitylation mechanisms (Kuo et al, 2004; Pollice et al, 2007). Moreover, it has been observed that in primary cells ARF turnover is very fast but, conversely, ARF protein appears to be very stable in cancer cells where proteasome degradation is severely impaired (Chen et al, 2010; Kuo et al, 2004).

In this work we provide evidences showing for the first time that PKC activation mediates phosphorylation and stabilization of ARF endogenously expressed protein. We also showed that an ARF mutant

mimicking the phosphorylated status of the protein, despite its ability to stabilize p53 and bind to MDM2, is impaired in blocking cell proliferation in both human and mouse cell lines. We, thus, identified a novel transcription independent mechanism that regulates ARF protein levels both in immortalized and cancer cells.

The hypothesis that ARF activity could be regulated by phosphorylation events has been suggested by several and independent observations. The Death Associated Protein Kinase (DAPK) is involved in the activation of ARF/p53 axis (Raveh et al, 2001). Interestingly, it has been described that DAPK is not able to induce the increase of p53 and p21 protein levels in p19ARF^{-/-} MEF cells, suggesting ARF involvement in this pathway. In addition, in vitro kinase assay showed that p19ARF can be phosphorylated by DAPK but both the phosphorylation sites and the physiological relevance of this phosphorylation remained unaddressed. DAPK, a calcium/calmodulin (CaM)-regulated Ser/Thr protein kinase, was originally identified as a factor that regulates apoptosis in response to the death-inducing cytokine signal interferon γ (INF- γ). In addition to its well-documented role in the regulation of apoptosis, DAPK may also play a role in survival pathways, reflected in its activation by growth factor signalling pathways and its ability to counteract tumor necrosis factor (TNF)- mediated apoptosis (Jin et al, 2002; Lin et al, 2010; Stevens et al, 2009). Furthermore, it has been reported that DAPK also has autophagic signalling activity, which can result in both survival or cell death (Lin et al, 2010). More recently, the hypothesis of the involvement of phosphorylation in controlling ARF activities has also been suggested by di Tommaso and colleagues (2009). ARF protein sequence has different potential target sites for phosphorylation such as Threonine (T8, T31, T73, T95) and Serine (S52, S64, S92, S127) residues. It has been reported that, specifically, substitution of Thr31 in the T31A mutant severely impaired p14ARF's ability to maintain chromosomal stability in p53-null cells and markedly enhanced its stability in p53-positive cells. In addition, the A41S mutant also displayed an

inefficient ability to stabilize p53 and inhibit DNA synthesis despite the same or greater expression compared to wild type p14ARF. Although these data suggested a potential phosphorylation of ARF protein, the authors were not able to confirm this hypothesis after transfection of p14ARF in U2OS cells (di Tommaso et al, 2009).

In addition to these evidences on ARF potential phosphorylation, our *in silico* examination of p14ARF protein sequence revealed three potential target sites for phosphorylation mediated by Protein Kinase C (PKC). In agreement with this, our data showed that PKC phosphorylates ARF *in vitro* and that these two proteins interact in different cell lines. Moreover, TPA-induced PKC activation resulted in the stabilization of cytoplasmic ARF.

It has been reported that both activation and downregulation of specific PKC isoforms is linked to tumor promotion and progression (Blobe et al, 1994; Chen and Mochly-Rosen, 2001; Murriel and Mochly-Rosen, 2003; Ron and Kazanietz, 1999). The combined effects of the various PKC isoforms can result in diverse conflicting consequences. The final outcome depends on tissue specificity, the levels of PKC expression, and the relative contribution of the different PKC isoforms in a given cellular context. The involvement of PKC in tumor progression has, thus, gained recognition as potential therapeutic targets for the treatment of various malignancies. These data are based on the observation of xenograft tumor growth inhibition by PKC- α knockdown in stomach, lung, bladder and colon cancer cell lines (Bosco et al, 2011), and increased tumor size from MCF-7 breast cancer cells overexpressing PKC- α , thus indicating a functional requirement for PKC α in tumorigenesis.

We, thus, decided to analyze the connection between ARF phosphorylation and its biological activity and stability. For first we demonstrated that ARF is phosphorylated in H1299 cells using immunoprecipitation experiments of ARF protein followed by immunoblotting with anti p-Threonine antibody. In addition, following TPA treatment we observed an increase of an ARF phosphorylated form. These data seem to suggest a role of phosphorylation in

the regulation of ARF stability. Interestingly, in line with the observation of di Tommaso and colleagues (2009), we failed in detecting ARF phosphorylation in U2OS cells also upon TPA treatment (unpublished data). This could suggest that ARF post transcriptional modification program could depend on the cellular context analyzed.

Among the three potential target sites for PKC mediated phosphorylation, the Threonine in position 8 lies within the 2-14 conserved region required for ARF biological functions and for its localization. Therefore, we functionally characterized its role in these ARF functions. We observed that mutation of Threonine 8 can influence ARF biological activity, stability and cellular localization. In particular the T8A mutant, which cannot be phosphorylated, although showing a reduced half-life, retains the same characteristics of the wild type protein such as the subcellular localization and the negative control of cell proliferation. On the other hand T8D mutant, that mimics a constitutive phosphorylated status of the protein, has similar stability of wild type but different cellular localization and activity. In fact, T8D has a predominantly nuclear/cytoplasmic localization and although it stabilizes p53 and efficiently binds MDM2, it is not able to efficiently control cell proliferation. These results suggest that the partial loss of function observed with the T8D mutant could be ascribed to p53 independent ARF activities specifically impaired by the T8D mutation.

Interestingly, it has been recently shown (Vivo et al, 2015) that mimicking ARF phosphorylation impairs MDM2 mediated ARF degradation by the proteasome. In addition, MG132 treatment induces ARFT8A accumulation in the cytoplasm indicating that the reason why ARF is not usually detectable in this subcellular compartment resides in a more rapid turnover.

These evidences led us to suppose that upon PKC activation, ARF levels increase and it localizes prevalently in the cytoplasm where it cannot properly explicate its growth inhibitory functions (figure 18).

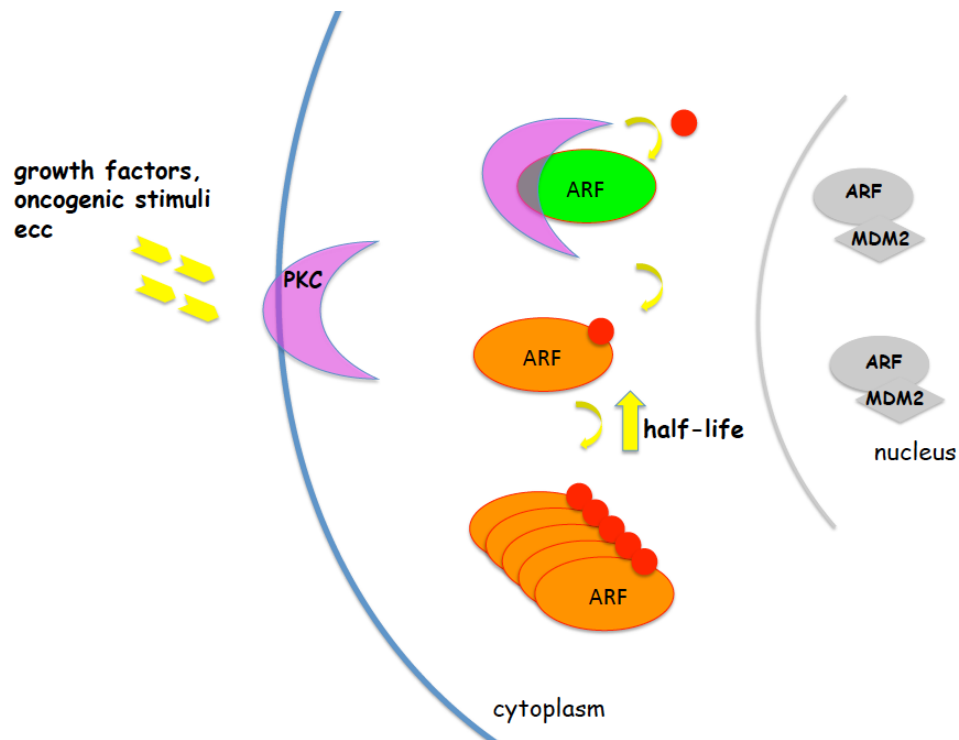


Figure 18. Schematic view of the hypotetic PKC/ARF connection.

After oncogenic insults PKC is activate and interacts with ARF in the cytoplasm. In this cellular compartment, ARF is phosphorylated and stabilized by PKC. This event keeps ARF from entering in the nucleus and inhibiting cell proliferation.

Increasing phosphorylation could, thus, be a strategy that cells orchestrate to promptly escape ARF growth suppression functions either in physiological conditions or when cells are forced to proliferate during cancer progression.

On the other side, these data might also indicate that ARF phosphorylation in tumor cells could be a mean to sustain tumor progression by conferring pro-survival properties to the cells.

In line with this hypothesis, recently, it has been reported that in cancer cells ARF is very stable compared to normal cells. In addition, it has been observed a pro-proliferative ARF role in tumor environment that seem to suggest ARF different roles depending on cell context. For example, it has been demonstrated that ARF silencing can limit the progression of mouse lymphoma suggesting its potential pro-survival function (Chen et al, 2009; Humbey et al, 2008; Xie et al, 2014). Further experiments are needed to clarify the molecular mechanisms underlining these observations and their relevance for cancer development in vivo.

In particular, we reasoned that the presence of ARF in the cytoplasm could results in partially unknown functions. On this basis we, thus, started to investigate ARF's role in several cell lines performing ARF knock down. In line with this new findings, our data point to a potential pro-survival role of ARF in both tumor (Hela) and stabilized cell lines (HaCat).

We unexpectedly observed loss of viability and induction of apoptosis upon ARF silencing both in Hela and HaCat cell lines. In addition, we observed that DAPK is involved in this process. In fact, we obtained a rescue of apoptosis upon simultaneous ARF and DAPK silencing in Hela cells. Moreover, given that DAPK can induce apoptosis via p53 activation, we also analyzed p53 protein levels in ARF depleted Hela cells. Although not irreversibly, in these cells the p53 pathway is disable due to enhanced protein degradation mediated by the viral protein E6. In line with this, ARF overexpression has no effect on cellular proliferation (Wesierska-Gadek et al, 2002). Nevertheless, it has been reported that a de-regulation of PKC δ in

cisplatin resistant HeLa cells may allow stabilization of p53 by DNA damage (Johnson et al, 2002). Interestingly, we observed an increase of p53 levels, suggesting that induction of apoptosis by DAPK could be p53-dependent. In agreement with these evidences, we were not able to observe induction of apoptosis upon ARF silencing in H1299 where p53 is mutated.

In line with these data, recent studies showed that ARF in spermatogonia facilitates meiotic progression in mice male germ cells. In particular, it has been demonstrated that ARF^{-/-} mice display a reduced sperm number compared to wild type mice with increase of p53-dependent apoptosis during germ cell development. Therefore, ARF expression in spermatogonia instead initiates a salutary feed-forward program that prevents p53-dependent apoptosis, contributing to the survival of meiotic male germ cells (Churchman et al, 2011).

Interestingly, during ARF silencing experiments we observed that ARF depleted cells showed a round phenotype. This phenomenon was not rescued after treatment with Z-VAD suggesting that the round morphology of ARF silenced cells was not a consequence of apoptotic program engagement.

This observation opens the way to the novel hypothesis that cytosolic (phosphorylated) localized ARF may be involved in cell spreading. In line with this hypothesis, unpublished data obtained in our laboratory have revealed that ARF interacts and co-localizes with the Focal Adhesion Kinase (FAK), a protein tyrosine kinase localized at sites of focal contacts with a role in cell spreading and migration. Interestingly, FAK expression and above all its phosphorylation on tyrosine 397, has been strongly correlated with tumor aggressiveness (Abbi and Guan, 2002).

One of cancer cell features is the alteration of adhesion process. In fact, transformed cells need to loss contact inhibition to growth without control. In addition, alterations in the adhesion pathway can affect cell migration. Moreover, both of these processes have a role in the activation of metastatic program. Metastasis is one of the clinical parameters with a strong

negative influence on the prognosis of cancer patients. Its detection in patients is usually associated with resistance to chemotherapeutic treatments and higher post-treatment recurrence states. Despite its malignancy the metastatic process is highly inefficient, it is estimated that less than 0.5% of cells entering the circulatory system form metastasis. Most cells die in non-permissive tissues, while others enter a dormant state in the peripheral niche, either at the single cell or micro-metastasis stage. When appropriate growth conditions arise, these cells exit the dormant state to form metastasis. Some studies suggested that autophagy by facilitating tumor dormancy may protect tumor cells from anoikis, a programmed cell death that occurs due to the loss of anchorage-dependent attachment to the extracellular matrix (ECM). Normal cells and tissues have low levels of basal autophagy that can dramatically increase upon growth factor deprivation, hypoxia, damaging stimuli and proteasome inhibition, promoting survival in the vast majority of cases. Recent studies have shown that ARF can induce autophagy (Pimkina et al, 2009; Reef et al, 2006). ARF involvement in autophagy could partly explain why ARF loss in some cancerous context can be detrimental for cancer development.

In addition, PKC has been implicated as a key molecule involved in cell spreading and migration (Timar et al, 1996), in part through interaction with beta1-integrins (Parsons et al, 2002). Cellular motility is sustained through assembly and disassembly of actin fibers at focal adhesions, whose composition and turn over are both controlled by PKC mediated phosphorylation (Boeckeler et al, 2010; Ivaska et al, 2005; Ng et al, 1999; Rosse et al, 2012).

Furthermore, it has been reported that DAPK exerts apoptotic effects by suppressing integrin functions and integrin-mediated survival signals, thereby activating a p53-dependent apoptotic pathway. It has also been observed that integrin or FAK activation blocks DAP-kinase-induced upregulation of p53 (Wang et al, 2002). Moreover, in an experimental metastasis model, the expression level of DAP-kinase is inversely correlated with the metastatic

activities of the tumors. The re-introduction of DAP-kinase into the highly metastatic tumors suppresses their ability to form metastases in mice, implying a role of DAP-kinase in anoikis (Inbal et al, 1997).

Therefore, further experiments will be required to clarify better the role of ARF protein in the cytoplasm and the molecular mechanisms connecting PKC, ARF and FAK not only in cell cultures but also in human tumor samples.

It will be interesting, also, to investigate the role of FAK in ARF-dependent morphology changes, viability and invasion using Hela cells silenced for both ARF and FAK. In addition, it will be intriguing to analyze the time window in which ARF expression is required in the acquisition of metastatic phenotype using stable ARF depleted inducible cell lines. Furthermore, it would be extremely interesting to analyze the effect of ARF depletion both in metastatic traits and in tumorigenesis using xenograft experiments.

6. CONCLUSIONS.

Collectively, our data suggest that in a tumor environment, after PKC activation, ARF protein is stabilized by phosphorylation and accumulates in the cytoplasm. In this cellular compartment, phosphorylated ARF is unable to efficiently block cell proliferation.

These data could be interpreted as a way of cancer cells to escape ARF surveillance in tumorigenesis, but they might also indicate that ARF phosphorylation in tumor cells could confer a pro-survival properties to the cells during cancer progression.

Experiments still in progress are now exploring the hypothesis that through the interaction with un-conventional molecular targets, such as the Focal Adhesion Kinase, ARF could mediate cellular response during cancer progression ultimately resulting in the regulation of adhesion and spreading.

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Mimicking p14ARF Phosphorylation Influences Its Ability to Restrain Cell Proliferation

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Abstract

The INK4a/ARF locus on the short arm of chromosome 9 is one of the most frequently altered loci in human cancer. It is generally accepted that ARF is involved in oncogenic checkpoint pathways by sensitizing incipient cancer cells to undergo growth arrest or apoptosis through both p53-dependent and independent pathways. While intensive studies have been focused on ARF activation at the transcriptional level, only recently mechanisms governing ARF turnover have been identified. Here, we show for the first time that p14ARF is a PKC target. Prediction analysis showed many potential phosphorylation sites in PKC consensus sequences within ARF protein, and, among them, the threonine at position 8 was the most conserved. Substitution of this threonine influences both ARF stability and localization. Furthermore, a phosphomimetic ARF mutation reduces the ability to arrest cell growth although the ability to bind MDM2 and stabilize p53 result unaffected. Thus we propose that phosphorylation of ARF in both immortalized and tumor cell lines could be a mechanism to escape ARF surveillance following proliferative and oncogenic stress.

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Introduction

The ARF protein is encoded by the alternative reading frame of the INK4a locus, one of the most frequent sites of genetic loss in human cancers. Over the last years, a direct contribution of ARF to tumor formation has been documented using genetic analysis of tumors, molecular and cell biology methods and animal models [1,2,3]. Both the human (p14ARF) and mouse (p19ARF) proteins function as important sensors of hyper-proliferative stimuli acting to restrict cell proliferation through both p53-dependent and independent pathways [4,5]. Under normal conditions the p53 protein is kept at low levels by its rapid turn over, and exists in a latent and inactive form. Activation of p53 in response to cellular stresses is mediated, at least in part, by inhibition of MDM2 functions [6]. In normal conditions, primary cells contain low levels of ARF. However, when stimulated to proliferate by oncogenic proteins such as Myc, E2F, E1A, oncogenic Ras and v-Abl [7,8,9,10], ARF levels increase. This phenomenon is generally accompanied by a parallel disruption of the inhibitory interaction between Mdm2 and p53, resulting in the accumulation of transcriptionally active p53 that induces specific responses such as cell-cycle arrest or apoptosis [11,12]. However, genetic analysis of tumors and the evidence that ARF can induce cell-cycle arrest in cells lacking Mdm2 and p53 support the notion that ARF might act independently of MDM2 and p53 [2,5,13,14,15]. Furthermore, the discovery of a plethora of ARF interactors and the observation that also viral, genotoxic, hypoxic and oxidative stresses activate an ARF-dependent response, suggest that ARF

has a wider role to protect the cell [16]. Given its strong ability to block growth and proliferation, cells must develop mechanisms that promptly reduce either its expression or functions when its activity is no longer required. While ARF activation, mostly occurring through transcriptional activation, has been the focus of intensive studies [17,18,19,20,21] only recently the mechanisms regulating ARF protein turnover started to be elucidated. ARF degradation can be regulated by the proteasome by both ubiquitin independent [22] or lysine-independent N-terminal ubiquitylation mechanisms [23]. Recently, a specific ARF ubiquitin ligase, ULF has been identified [23,24]. Moreover, it has been observed that in primary cells ARF turnover is very fast: these cells express very low levels of protein, which could be promptly stabilized upon proteasome inhibitor treatment. Conversely, ARF protein appears to be very stable in cancer cells where proteasome-mediated ARF degradation is severely impaired [24].

It has been reported that Protein Kinase C alpha (PKC α) is involved in the stabilization of exogenously expressed ARF protein levels [25]. Multiple PKC isoforms have been associated with epidermal keratinocyte differentiation program and some of them are activated by calcium [26]. We have previously shown that both p14ARF transcript and protein levels increase upon calcium-induced keratinocyte differentiation. Here we present data showing that endogenous human ARF protein levels increase following stimuli known to activate the PKC pathway in both tumor and immortalized keratinocyte cell lines. A careful examination of ARF protein sequence revealed three potential PKC phosphorylation sites. Among them, Threonine at position 8

lies within the most conserved region encompassing amino acids 2–14 and is required for the activity and nuclear localization of ARF protein [26,27,28]. Our data indicate that mutation of this residue, mimicking a phosphorylation status of the protein, influences both ARF activity and cellular localization.

Results

p14ARF Protein Levels Increase following Calcium and TPA Treatment

We had previously shown that, during calcium induced differentiation of transformed human keratinocyte cells (HaCaT), p14ARF increases both at protein and transcriptional levels [29]. To understand if the increase in ARF levels was due to a transcription independent mechanism, HaCaT cells were transfected with increasing amounts of a plasmid expressing p14ARF and treated with 2mM calcium for 12h in presence of serum. Western blot analysis of total protein extracts (Fig. 1a) shows that ARF levels increase following stimulation. Quantification of multiple experiments shows a two fold increase of ARF protein levels following calcium treatment (2 ± 0.5). It has been reported that PKC α , which can be activated by Ca²⁺, is involved in TPA-mediated stabilization of p14ARF [25]. Thus we analysed ARF protein levels in HaCaT cells treated with 10 μ M TPA (Fig. 1b). The experiment shows that TPA induces a fast PKC activation, followed by an increase of ARF protein levels after 10 minutes of treatment (2.5 ± 0.09). Accordingly, a decrease of almost 70% ARF levels (0.3 ± 0.05) was obtained when cells were grown in presence of 5 μ M bisindolylmaleimide, a known inhibitor of PKC activation (Fig. 1c). Similar results were obtained using the p53 deficient human lung cancer cell line H1299 expressing endogenous ARF (data not shown). Altogether, these experiments prompted us to further investigate on the potential role of PKC and phosphorylation on p14ARF regulation.

p14ARF Interacts with and is Phosphorylated by PKC in vitro

Analysis of ARF protein sequence with GPS 2.1 [30] software revealed the presence of three potential PKC phosphorylation sites. Among these, Threonine 8 and Serine 52 lie in the functional active domain of the protein, while Serine 127 in the C-ter region of the protein (Fig. 2a). Interestingly, threonine 8 is conserved among all ARF orthologs. We thus performed an in vitro assay to test ARF ability to be phosphorylated by PKC. Phosphorylation assay was performed using a purified MBP::ARF protein and a commercially available Protein Kinase C phosphorylation assay. A reaction containing only the maltose binding protein was performed in parallel, as negative control. Autoradiography showed a clean phosphorylated MBP-ARF signal, indicating that ARF is actually a PKC substrate (Fig. 2b, compare lane 2 with lane 1). A fusion protein in which the three PKC consensus sequences were simultaneously mutated was used as control. This mutant was not phosphorylated in these experimental conditions, thus confirming the specificity of the kinase assay (data not shown). As many PKC substrates are dephosphorylated by PP1 [31], equal aliquots of the reaction, after kinase incubation, were subjected or not to a dephosphorylation assay using the Protein Phosphatase-1 (PP1) catalytic subunit. As shown in Fig2, panel B, the phosphorylated ARF signal disappeared upon PP1 treatment (compare lane 4 with lane 3). The ability of PKC to phosphorylate a p14ARF mutant where the threonine 8 was replaced with an alanine (MBP::p14ARF T8A) was also analysed. As shown in Fig. 2c this mutant is still a PKC substrate even if at lesser extent than the wt protein.

We next verified the physical association between ARF and PKC by coimmunoprecipitation experiment in H1299 cells expressing endogenous p14ARF (Fig. 2d). As a negative control we used the human osteosarcoma U2OS cell line that is wild type

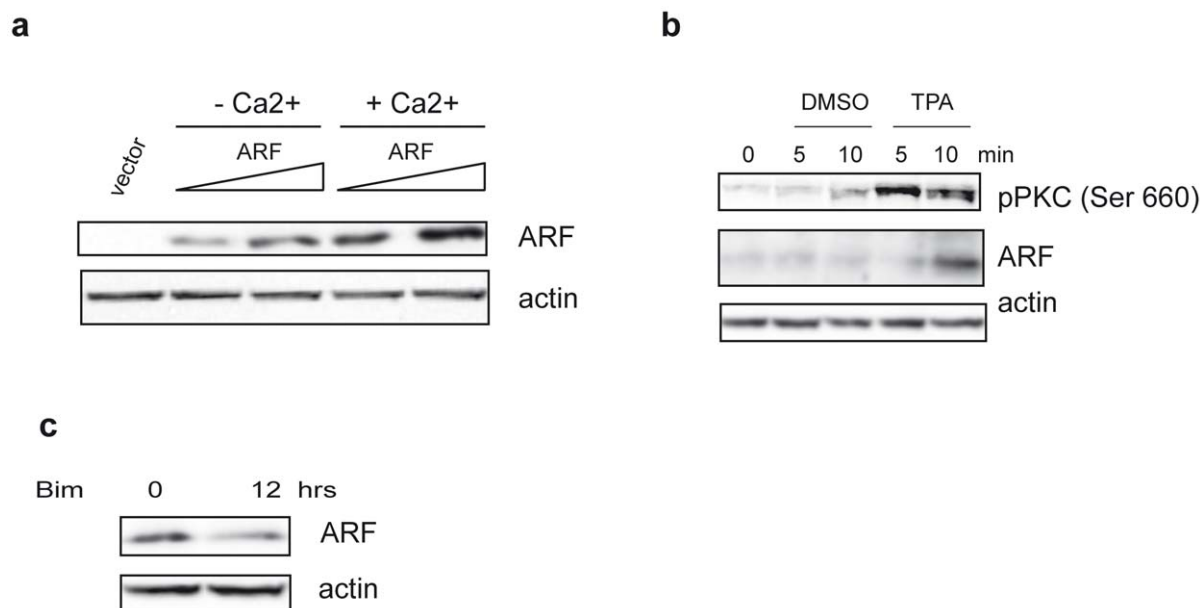


Figure 1. SDS-PAGE analysis of ARF protein levels. a) HaCaT cells were transfected with an empty plasmid (vector) and increasing amount of p14ARF expression plasmid. Twenty-four hours after transfection cells were treated or not with 2mM Ca²⁺ as indicated. Cellular extracts were immunoblotted and analysed with anti His antibody to detect exogenous ARF levels and anti actin as loading control. b) HaCaT cells were treated with 10 μ M TPA for different time points. Protein extracts were subjected to WB with anti ARF (C-18) and an anti pPKC pan antibody that recognizes all the PKC isoforms phosphorylated at a carboxy-terminal residue homologous to serine 660 of PKC β II (activated pPKC). Actin was used as loading control. c) HaCaT cells were treated with 5 μ M Bisindolylmaleimide (Bim) for 12 hours (hrs) and analysed with anti ARF and anti actin antibodies. doi:10.1371/journal.pone.0053631.g001

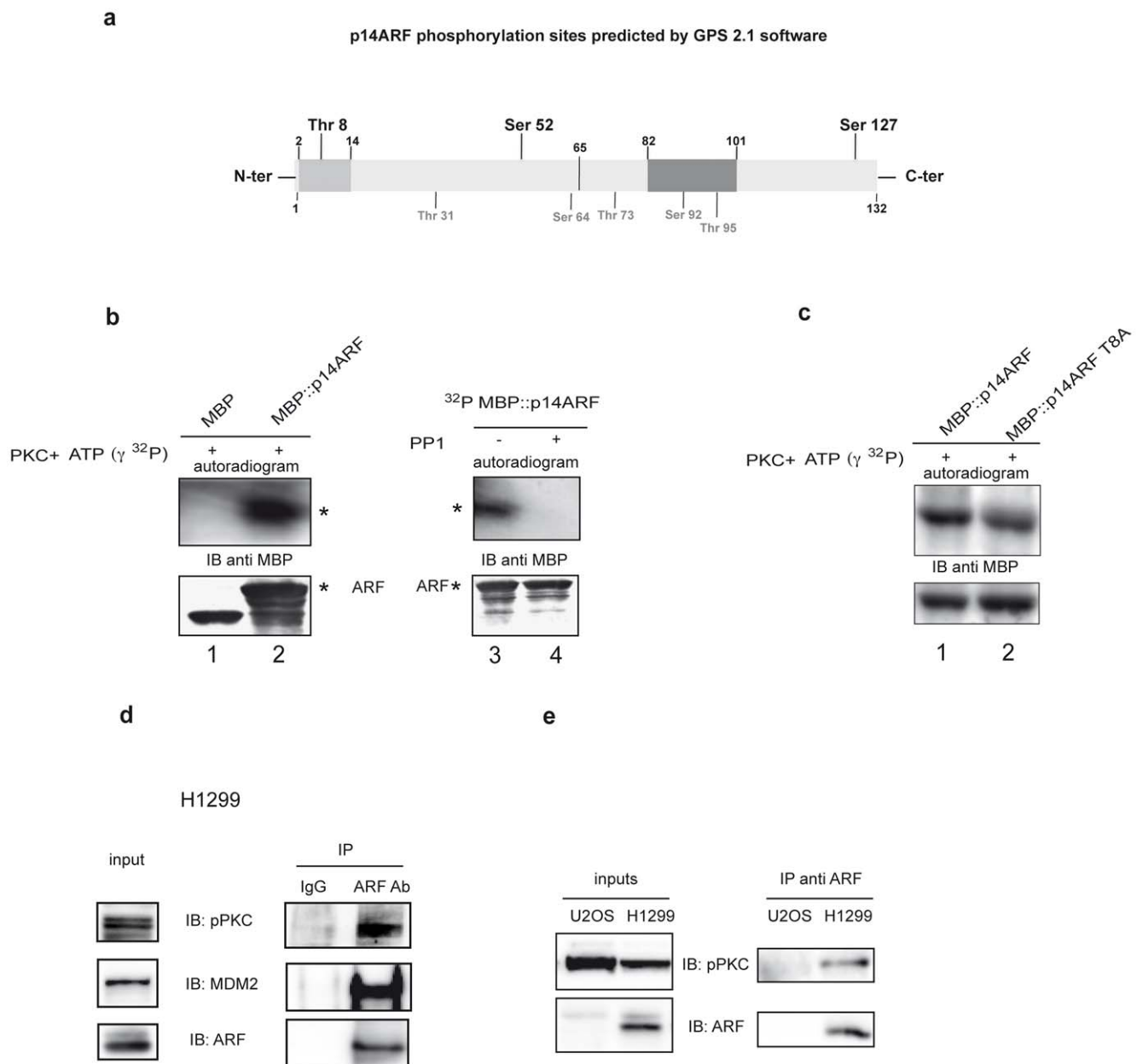


Figure 2. ARF is a substrate of PKC and PP1 in vitro. a) Diagrammatic scheme of p14ARF phosphorylation sites identified in silico. In light gray is indicated the conserved 2–14 amino acid stretch within the N-terminal exon 1 β encoded domain (amino acid 1–65), while in dark gray the exon 2 nuclear localization signal. PKC phosphorylation sites are shown in bold above the scheme, while other kinases target sites are denoted in gray below the scheme. b) In vitro phosphorylation assay. MBP (lane 1) and MBP::ARF (lane 2) were incubated with labelled γ ATP and catalytic PKC subunit. Reactions were then subjected to SDS-page and WB followed by ON exposure (upper panel). The lower panel shows the same filter immunoblotted with anti MBP antibodies. Detections of MBP::ARF is denoted by (*) on the autoradiograms and WBs. Purified phosphorylated MBP::ARF was divided in two aliquots and incubated (lane 4) or not (lane 3) with catalytic PP1 and processed as described in a). c) In vitro phosphorylation assay of MBP::T8A mutant. d) Co-immunoprecipitation of pPKC with ARF: H1299 lysates were incubated with anti-ARF antibody or with mouse IgG as negative control. Samples were analyzed by immunoblotting with anti pPKC Ser660, anti ARF and anti MDM2 antibodies.

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for p53 but lacks expression of p16 and p14ARF proteins. Total extracts were immunoprecipitated with anti ARF antibody and complexes probed with anti ARF and anti phospho-PKC pan antibody. Protein complexes were also assayed with an antibody recognizing a well known ARF protein partner, the MDM2 oncoprotein, as positive control. As shown in Fig. 2 (panel d and e), a complex between ARF and activated PKC is exclusively detected in H1299 cells.

Mutation of Threonine 8 does not Affect ARF Folding

The conserved threonine at position 8 lies in a twelve amino acid stretch (region 2–14, see Fig. 2a) strictly required for ARF biological activity. To analyse the role of this residue in ARF functions, we constructed ARF mutants where threonine 8 was replaced either by an alanine residue, that cannot be phosphorylated (T8A mutant), or by an aspartic acid (T8D mutant) that mimics the phosphorylated status of the protein. To rule out the

possibility that the inserted mutations could have resulted in an incorrectly folded protein, we tested the binding to MDM2, interacting with the N-terminal ARF region [28], and to p32 that binds ARF through the C-terminal domain [32]. U2OS cells were transfected with expression plasmids encoding human MDM2 or myc tagged p32, and/or X-press tagged wild type or mutant ARF proteins (Fig. 3). Immunoprecipitated complexes were blotted and probed with anti-MDM2, anti-myc and anti-ARF antibodies. The experiments show that the substitution of threonine at position 8 does not affect ARF binding to MDM2 and p32 (Fig. 3a and b).

Mutation of Threonine 8 Affects ARF Protein Turn-over

Immunoblots of total cell lysates reproducibly showed lower levels of T8A mutant compared to wt and T8D mutant suggesting lower protein stability. To explore this possibility, half-life studies of the wt and mutant proteins were performed using the protein synthesis inhibitor cycloheximide (Fig. 4). Half-life analyses showed that reduced expression of T8A mutant correlated with decreased stability. Wild-type ARF showed a half-life of 6 hours, as previously reported [33]. While T8D mutant stability is compa-

rable to that of the wt, the half-life of the T8A mutant is reduced to 2 hours, indicating that this residue could be involved in p14ARF stability (Fig. 4a and 4b).

Mimicking Thr8 Phosphorylation Induces ARF Accumulation in the Cytoplasm and Nucleus

It has been extensively shown that ARF amino acid stretch 2–14 is not only involved in MDM2 binding and ARF mediated cell cycle arrest but also contribute to p14ARF nucleolar compartmentalization [28,33]. Thus we analysed the effect of T8 mutations on ARF subcellular localization following transfections in U2OS cells.

As expected, following transfection wt ARF protein presents different localization patterns with cells showing three different stainings: nucleolar (Fig. 5a panel i), nuclear (Fig. 5a panel ii) and nucleo-cytoplasmic (Fig. 5a panel iii). Quantitation of this result showed, for the wt protein, a clear nucleolar and nuclear distribution pattern in the majority of transfected cells (90%), and a nuclear-cytoplasmic distribution in the remaining 10% of cells (Fig. 5b). The T to A substitution did not significantly

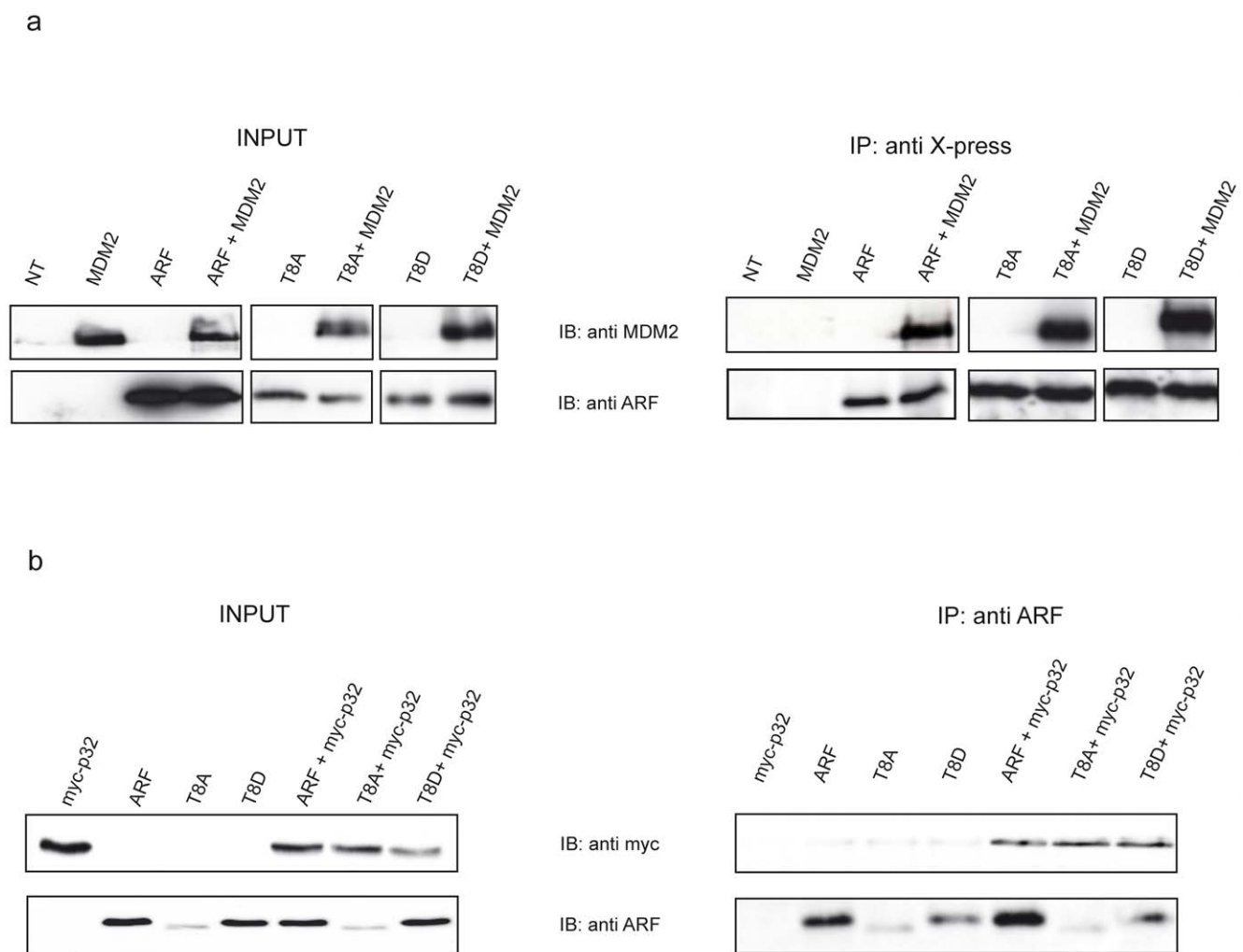


Figure 3. Mutation of Threonine 8 does not affect ARF folding. a) U2OS cells were transfected with plasmids encoding X-press tagged version of either wild type or mutants ARF proteins alone or in combination with plasmid encoding human MDM2 protein. Cellular lysates were immunoprecipitated with anti X-press antibody and samples analyzed by immunoblotting with anti MDM2 and ARF antibodies. b) cells were transfected with myc tagged p32 encoding plasmid and wt and mutants ARF as described in a). Cellular lysates were immunoprecipitated with anti ARF and analysed by IB with anti myc and anti ARF antibodies.
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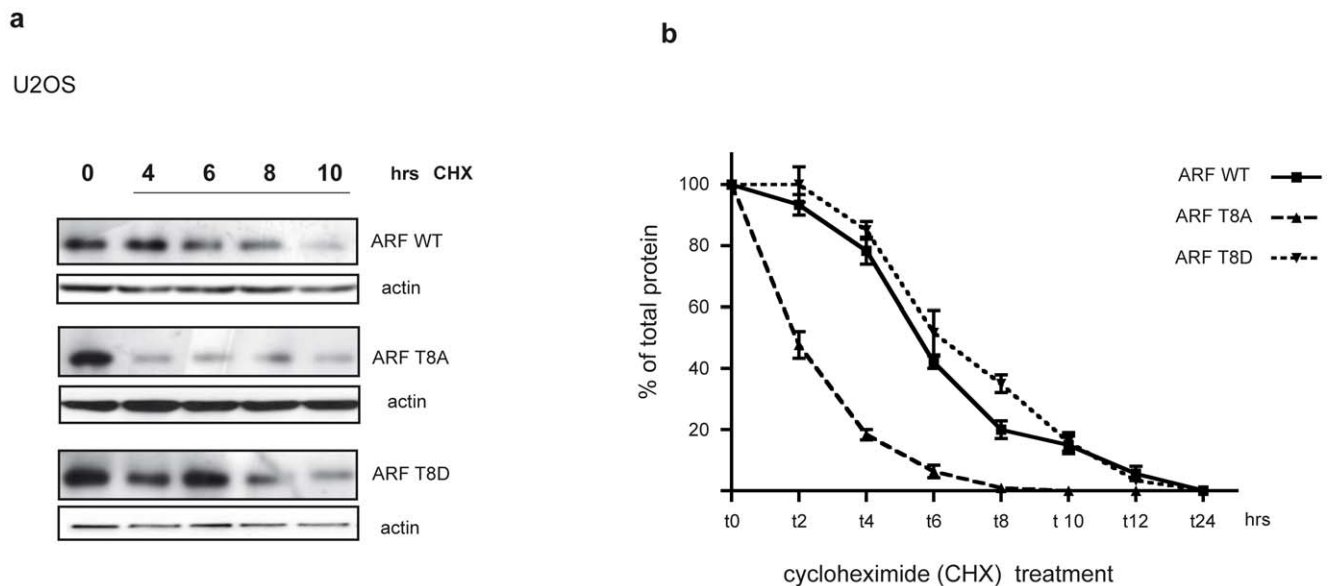


Figure 4. Protein turn-over analysis. a) Half-life analysis of the wt and mutated p14ARF in human U2OS cells. Cells were transfected with p14ARF wt, T8A and T8D plasmids and treated with cycloheximide (CHX) for the indicated times two days after transfection. ARF and actin levels were analysed by WB of total extracts. b) The plot represents half-life analysis of wt and mutant ARFs. Band intensities were quantified by Image J analysis and actin normalized before being plotted in graph. The amount of protein at different time points is expressed as percentage of total protein, i.e. protein amount at t0. Each profile represents the mean of three independent transfections and WB experiments. Standard deviations are also shown. doi:10.1371/journal.pone.0053631.g004

compromise the number of cells showing a nuclear localization as shown by quantitation in Fig. 5b. However, replacement of the same residue with an aspartic acid dramatically reduced the proportion of cells showing exclusively nucleolar ARF localization and led to an increase in the percentage of cells with nuclear and nuclear-cytoplasmic ARF staining (Fig. 5b). Experiments performed with GFP fusion proteins gave similar results (data not shown). These data suggest that a mutation mimicking T8 phosphorylation can induce accumulation of the protein both in the cytoplasm and in the nucleus.

Cytoplasmic Localized ARF Protein is Phosphorylated

Endogenous ARF localization was then analysed in tumor cells such as H1299 by western blot of cytoplasmic and nuclear extracts. Efficient fractionation was checked with anti PARP-1 for nucleus and anti actin or RACK-1 antibodies for the cytoplasm as these proteins are mainly enriched in these cellular compartments (Fig. 6a and [34]). Using different ARF antibodies, we consistently found an ARF immunoreactive band both in nuclear and cytoplasmic extracts (Fig. 6a and data not shown). Moreover, fractionation experiments, performed in the HaCaT cell line, showed ARF expression almost exclusively in the cytoplasm (Fig. 6a right panel), in agreement with our previously published immunofluorescence data [29]. Western blot analysis also shows that activated PKC is mainly localized in the cytosol (Fig. 6a).

We next looked at the effect of TPA addition on the ARF protein levels both in nuclear and cytoplasmic compartments. As shown in Fig. 6b, following TPA treatment, only the cytoplasmic pool of ARF increased. Consistently, pPKC appeared upregulated in the cytoplasm. We thus decided to check the phosphorylation status of cytoplasmic ARF by a lambda phosphatase treatment (Fig. 7a). Equal amount of cytoplasmic extracts were incubated or not with lambda phosphatase and subjected to SDS-page and western blot with anti ARF antibody. The experiment clearly

shows an increase of the ARF band in the treated sample (compare lane+with-) in both cell lines and using different ARF antisera (data not shown).

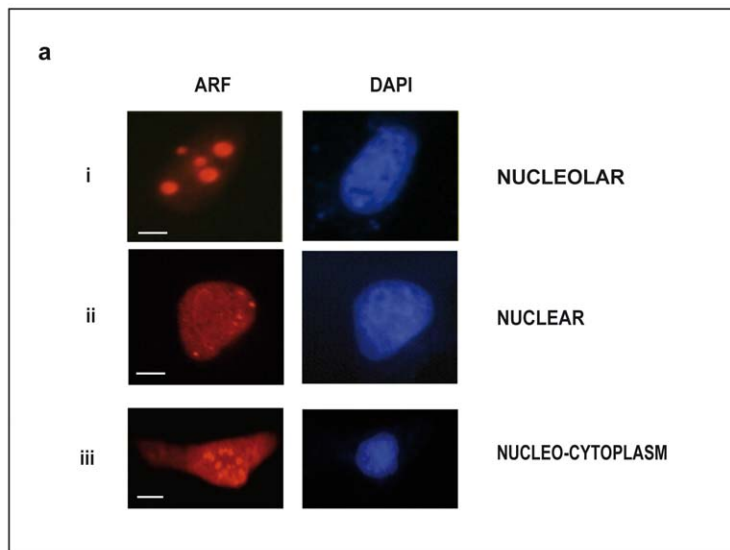
We next assessed ARF phosphorylation status in H1299 cells after TPA stimulation using a different approach. Cells were treated with TPA and protein extracts subjected to immunoprecipitation with anti ARF antibody followed by immunoblot with an antibody directed against phosphorylated threonine (pThr antibody). The experiment shows a 15 KDa band in untreated sample whose intensity increases following TPA stimulation (Fig. 7b upper panel). Identity of this band was confirmed by western blot with anti ARF antibodies (Fig. 7b lower panel).

Mimicking ARF Phosphorylation Inhibits ARF Biological Activity

It has been repeatedly shown that ARF ectopic expression inhibits cell proliferation at least in cells expressing wt p53 [33,35,36]. Wild type and mutant ARF ability to block proliferation was tested by Colony formation efficiency assay (CFE assay) in U2OS cells where p53 pathway is intact. Results showed that, although less stable, T8A mutant was able to block cell proliferation with the same efficiency as the wt (Fig. 8a). Ectopic expression of the T8D mutant was instead less efficient (60% residual colonies after G418 selection in T8D versus 30% of wt and T8A, Fig. 8a). Similar results were obtained in the ARF null NIH 3T3 mouse cells harbouring wt p53 (Fig. 8a). Interestingly, western blot assays show that T8D ectopic expression is efficient in stabilizing p53 (Fig. 8b).

Discussion

In this paper we report data showing that two known inducers of PKC activation, Ca^{2+} and TPA, both induce an increase of p14ARF protein level in immortalized and in tumor cell lines. The PKC family consists of at least ten serine/threonine kinases playing a central role in cell proliferation, differentiation, survival



b

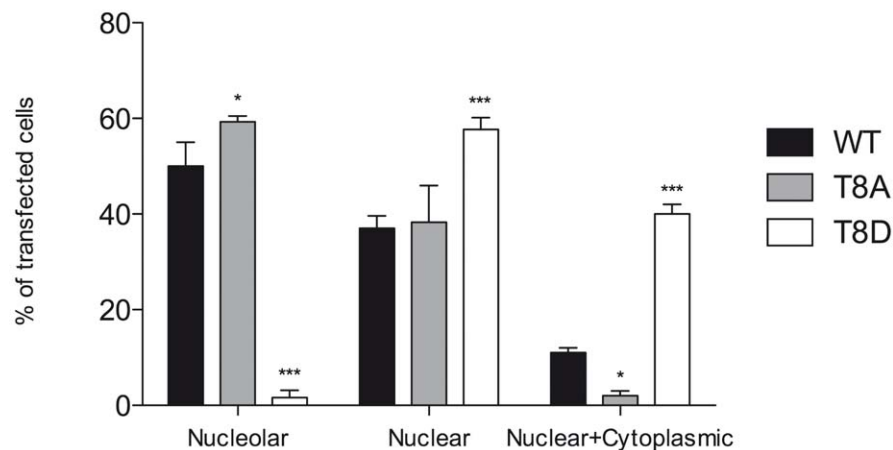
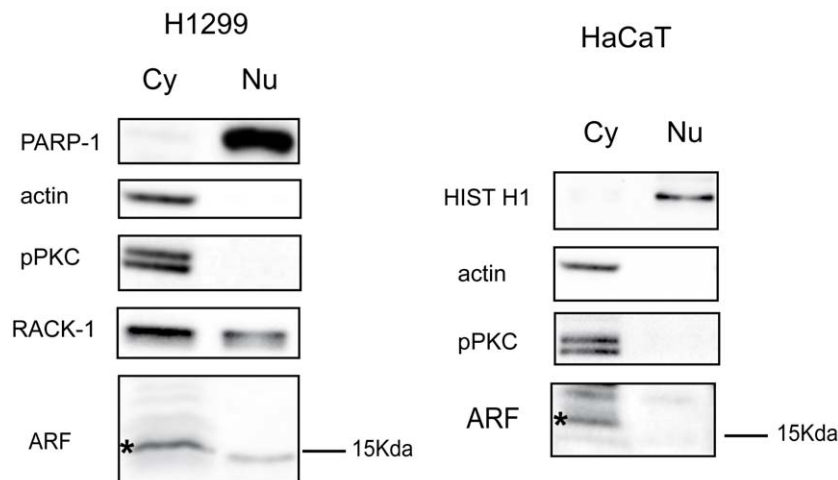


Figure 5. ARF localization analysis. U2OS cells were transfected with p14ARF expression plasmids (wt or mutants) and visualized by IF with anti histidine antibody. Nuclei were stained with DAPI. Images were taken with a Nikon fluorescent microscope. a) Visualization of the three different wt ARF localization patterns are indicated with i, ii and iii. Scale bar: i, ii = 3 μ m; iii = 7 μ m. b) The histogram, representing the mean of three independent experiments, reports the percentage of transfected cells showing each localization pattern. Standard deviations are also shown. Asterisks indicate statistically significant differences between the sample and wt ARF (* = $p < 0.05$; *** = $p < 0.001$). doi:10.1371/journal.pone.0053631.g005

and death [37]. While classical isoforms need both calcium and the lipid molecules Diacylglycerol (DAG) or phorbol esters (TPA) for activation, novel kinases are calcium independent and lipid dependent, while atypical are both calcium and lipid independent. Our results suggest that the PKC isoform(s) involved might belong to the classical and/or novel subfamily. Accordingly, the involvement of PKC α in TPA-mediated stabilization of p14ARF has been reported [25]. PKC α , the major conventional Ca²⁺ responsive PKC in epidermis, is highly abundant in skin and is believed to play a key role in calcium induced keratinocyte differentiation [38,39]. Remarkably, defective differentiation in skin cancer correlates with elevated PKC α activity. The positive correlation between tumor promoting effects of various phorbol esters with their ability to activate certain PKC isoforms, indicates

that PKC activation is a critical step in skin tumor promotion [40]. Accumulating data show that PKC is linked to promotion and progression of several tumor types [41,42,43,44] and a number of pharmacological PKC inhibitors are developed and currently used in clinical trial. However, PKCs play either as oncogenes or tumor suppressors in human cancer development [45] and, therefore, the combined effects of the various PKC isoforms can result in diverse, conflicting consequences depending on individual isozyme, cell-type and subcellular localization.

Our findings that ARF is regulated through the activation of PKC pathway led us to the hypothesis that ARF function might be regulated by phosphorylation. ARF contains numerous potential phosphorylation sites, and one study suggested that it could be a target of death associated protein kinase (DAPK) [46].

a**b**

H1299

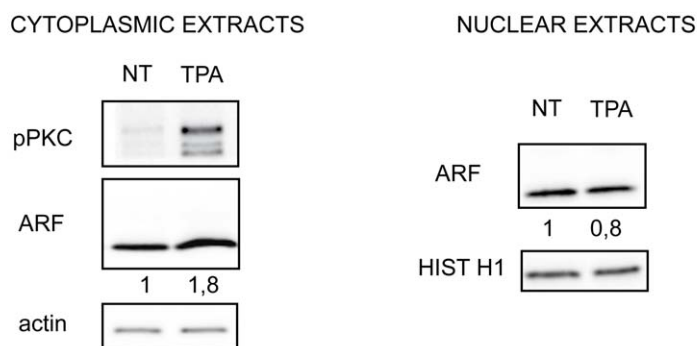


Figure 6. Sub-cellular fractionation. H1299 cells, endogenously expressing p14ARF, were subjected to subcellular fractionation. a) Left panel: cytoplasmic (Cy) and nuclear extracts (Nu) from equal number of cells were subjected to SDS-page and immunoblot with anti PARP-1 (nuclear), actin and RACK-1 antibodies (cytoplasm), anti pPKC and anti ARF antibodies. Right panel: HaCaT cells were subjected to subcellular fractionation and analysed as previously described. Histone H1 is a control of nuclear extracts. Cytoplasmic ARF species are indicated by an asterisk (*). b) H1299 cells were treated with TPA and subsequently subjected to subcellular fractionation. Equal amount of cytoplasmic (left panel) or nuclear (right panel) extracts were immunoblotted with anti ARF, pPKC, and actin and histone H1 as loading control for cytoplasm and nucleus respectively, as described. WB shown are representative of at least three independent experiments. Normalized ARF band intensities, shown below each corresponding band, are expressed as fold enrichment respect to untreated samples arbitrarily set to 1 (see material and methods for details). doi:10.1371/journal.pone.0053631.g006

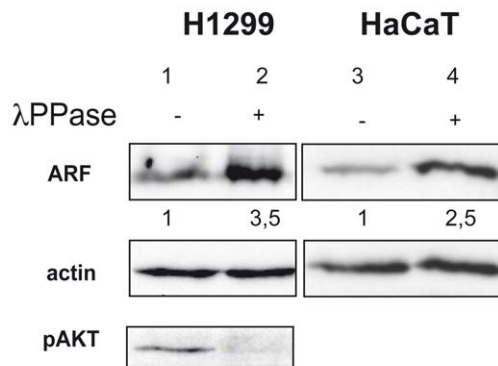
Moreover, the potential involvement of phosphorylation in controlling ARF activities has already been suggested by different experimental approaches [47,48].

Our hypothesis is supported by several findings. First, sequence prediction analysis shows that three PKC consensus sequences are present within ARF protein sequence, and in vitro kinase assay shows that the protein can be specifically phosphorylated by PKC and dephosphorylated by PP1. In agreement, a protein complex consisting of activated PKC and ARF exists in cells, and we observed that immunoprecipitated ARF can be recognized by a pThr antibody in the H1299 cell line (Fig. 6b). Moreover, following TPA treatment, the amount

of phosphorylated ARF species increase (Fig. 6b lower panel). Significantly, TPA-induced PKC activation results in the stabilization of cytoplasmic ARF.

Furthermore, we have functionally characterized the role of the conserved threonine 8 that lies within a PKC consensus sequence in ARF protein. While the T8A mutation does not affect ARF localization, the phospho-mimetic T8D mutation dramatically reduces ARF ability to be exclusively localized in the nucleoli inducing the protein to accumulate in the cytoplasm in a significant fraction of cells. The observation that λ phosphatase treatment results in a clear thickening of the ARF electrophoretic band, suggests that the cytoplasmic protein is indeed phosphor-

a



b

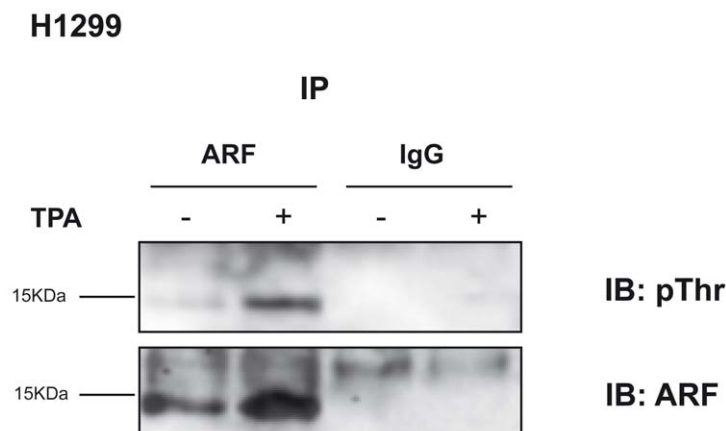


Figure 7. ARF phosphorylation assays. a) λ phosphatase assay: equal amount of HaCaT and H1299 cytoplasmic extracts were incubated (lane 2 and 4) or not (lane 1 and 3) with λ protein phosphatase (λ PPase), analysed by high resolving SDS-PAGE and blotted with anti ARF antibody. Actin is a loading control, while anti pAKT immunoblot is a reaction control. ARF band intensities, quantified as previously described, are also given. b) Immunoprecipitation: H1299 cells treated or not with TPA were immunoprecipitated with anti ARF antibody, subjected to SDS-page and blotted with anti pThr antibody (upper panel). The filter was stripped and incubated with anti ARF antibody (lower panel).
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ylated. On the basis of these results we speculate that multiple phosphorylated ARF species are present in the cytoplasm, and, after phosphatase treatment, converge to the 15Kd band, increasing its intensity. Accordingly, the MBPT8A ARF mutant is still a PKC substrate *in vitro* (Fig. 2c), suggesting that multiple sites could be phosphorylated within the endogenous protein, as indicated by *in silico* analysis. Finally, while the T8A mutant retains the ability to inhibit proliferation as the wt protein, the phosphomimetic mutant T8D is severely impaired in the control of cell proliferation. Interestingly, both MDM2 binding and p53 stabilization appear to be unaffected by the T8D mutation. These results are in agreement with the notion that ARF dependent p53 activation takes place in the nucleus [47,49], and suggest that the partial loss of function observed with the T8D mutant could be ascribed to p53 independent ARF activities specifically impaired by the T8D mutation. Our data also suggest that, at least in a p53

plus cell environment, the unphosphorylated protein is active in blocking cell proliferation.

Analysis of protein turnover shows that the T to A substitution strongly reduces protein half-life from six to two hours. Despite its short half-life, T8A retains both nucleolar localization and control of cell progression abilities. Given that it is expressed at very low levels respect to the wt protein, the experiments indicate that few active ARF molecules can guarantee an efficient control of cell proliferation [50,51].

Altogether our data suggest that, following activation of PKC, ARF protein is phosphorylated and accumulates in the cytoplasm where it is unable to efficiently control cell proliferation. Further experiments are needed to precisely identify the molecular pathway, the potential involvement of additional kinase/s and the ARF residues directly involved in this mechanism.

In conclusion we suggest that in a tumor environment, where PKC is activated, ARF levels increase but its growth inhibitory

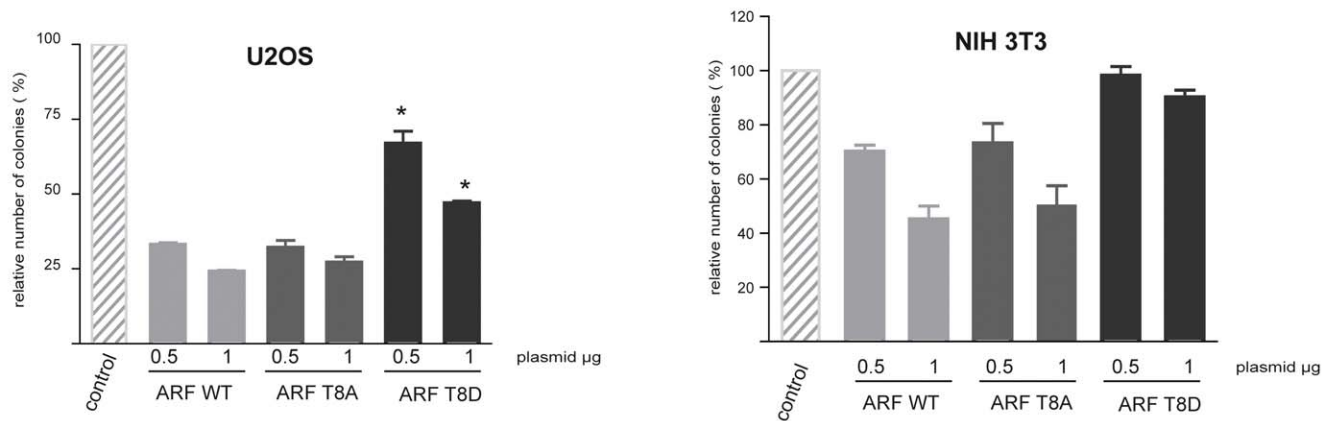
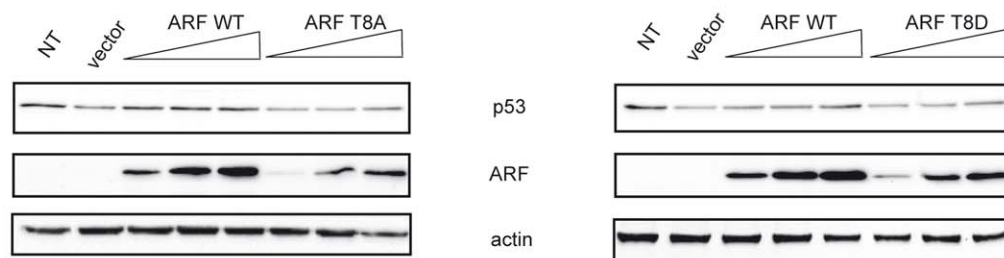
a**COLONY FORMATION ASSAY****b****p53 stabilization****U2OS**

Figure 8. Growth suppression by wt and mutant ARF proteins. a) Graph indicating ARF and mutants ability to inhibit cell proliferation. U2OS and NIH cells were transfected with the indicated amounts of ARF and mutant expression vectors. After two weeks selection to isolate transfected colonies, cells were fixed and stained with crystal violet. The plot represents the percentage of colonies obtained with the indicated plasmids relative to that detected with the empty vector. Values represent the mean of four independent experiments. Standard deviation for each value is also given. Asterisks (*) indicate statistically significant differences ($p < 0.001$) for the mutant samples compared to wt ARF. b) Western blot showing p53 stabilization following ARF (wt and mutant) transfection in U2OS cells.
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function is impaired. Interestingly, despite its role in growth suppression, ARF is overexpressed in a significant fraction of human tumors [52]. These observations, together with our findings underscore the need for a careful re-evaluation of the status and role of p14ARF in human cancer.

Our observations uncover a novel mechanism through which cancer cells might escape ARF surveillance during cancer progression. A deeper understanding of the PKC dependent ARF functions may provide previously unknown and critical therapeutic targets for cancer treatment.

Materials and Methods**Cell Culture, Cloning, Protein Expression and Reagents**

The human epithelial cell line HaCaT was obtained from Dr. Antonio Costanzo, Department of Dermatology, University of Rome Tor Vergata, 00133 Rome, Italy. All other cell lines (U2OS and H1299) were purchased from the American Type Culture Collection (ATTC). Cells were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum (Euroclone, Life Science) at 37°C in a humidified atmosphere of 5% (v/v) CO₂ in air. Cells were transfected using LipofectAMINE 2000 reagent (Invitrogen). For CFE

analysis U2OS were transfected with calcium phosphate precipitation as previously described [53].

The T8 amino acid changes were inserted into human p14ARF by site-directed mutagenesis using a wild-type p14ARF cDNA template. Standard polymerase chain (PCR) reaction conditions were used with each set of primers containing the desired mutations, and all products were verified by sequencing before subcloning into pMal, pcDNA3.1 and pEGFP vectors.

Antibodies used in this study: anti PARP-1 and anti-phospho-PKC (pan) antibody (Beta IISer660) (from Cell Signaling Technologies 9542, Boston, MA, USA); anti RACK-1 B3, anti ARF C-18, anti actin I-19, Anti Histone H1 N-16, anti p53 DO-1, anti MDM2 SMP-14, (from Santa Cruz); anti ARF 4C6/4 (Abcam); anti ARF Ab2 14PO2 (Neomarkers); 6XHis monoclonal antibody (Clontech); anti myc antibody 06549 (Upstate); anti X-press monoclonal antibody (Invitrogen); anti pThreonine Q7 (Qiagen). Western blot (WB) analysis was performed as previously described [29]. The molecular weight marker used for SDS page is the PageRuler™ Prestained Protein Ladder (Fermentas and Pierce). Extracts were blotted onto PVDF Immobilon-P transfer membrane (Millipore cat. NO. IPVH00010). Proteins were visualized with an enhanced chemiluminescence detection system (Amersham ECLplus™) and images were taken with ChemiDoc XRS System (Bio-Rad Laboratories) and analysed with the QuantityONE software except when differently indicated.

Reagents used in this study: TPA was purchased from AppliChem, Cycloheximide and Bisindolylmaleimide from Calbiochem. TPA and cycloheximide were dissolved in DMSO and used at 10 μM and 80 μg per ml respectively. Bisindolylmaleimide (Bim) was used at 5 μM final concentration. ARF band intensities following various treatments (Fig. 1–6–7) were quantified, normalized to actin (or to histone H1 in case of nuclear extracts in fig. 6) and reported as fold increase or decrease compared to untreated sample. Each value represents the mean of three independent experiments ± standard deviations.

Protein Turnover Analysis

For half-life studies, transfected U2OS were replated onto 6-well dishes, allowed to adhere overnight, and treated with cycloheximide or left untreated (0 hour control) for various time points. Cells were harvested and p14ARF protein levels examined by WB analyses of whole cell lysates with anti ARF antibody (C-18, Santa Cruz) and actin as loading control. Band intensities at the different time points were quantified by Image J Software, normalized to actin and reported in graph as percentage of total protein (protein a t0). Each profile represents the mean of three independent experiments. Standard deviations are also shown.

Subcellular Localization Assay

U2OS cells (5.0×10^4) were plated in 35 mm dish on micro cover glasses (BDH) and transfected with 0.5 μg of plasmid expressing wt or mutant ARF. At 24 hrs after transfection with the indicated vectors, cells were washed with cold phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde (Sigma-Aldrich, Germany) for 15 min at RT. Cells were permeabilized with ice cold 0.5% Triton X-100 for 5 min and then washed with PBS. Subcellular localization were determined by using the monoclonal antibody against 6XHis, 1h a RT, followed by incubation with a Cy3-conjugated anti-mouse antibody (ImmunoResearch Laboratory) at 37°C for 30 min. After PBS/0.05% Tween washing, the cells were incubated with DAPI (Sigma-Aldrich, Germany) for 3 min and washed with PBS/0.05% Tween. Coverslip were mounted with Vectashield

(VectorLab) and examined under a fluorescence microscope (Nikon). For each transfection point, subcellular localizations were analysed in 100–150 cells and results plotted in graph. Histograms (% of transfected cell ± S.D.) represent the mean of at least three independent transfection experiments. Statistical analysis of variance (Anova plus Bonferroni post test) was performed using GraphPad Prism 5.0 software.

Subcellular fractionation of H1299 and HaCaT cells was carried out as by Colucci-D'Amato et al., 2000 [54] with minor modifications. In order to analyse ARF protein distribution in the nuclear and cytoplasmic compartments, same number of cells and nuclei were processed as described. Cells were harvested, washed in ice cold PBS, resuspended in hypotonic buffer (Hepes 10mM, KCl 60mM, DTT 1mM plus protease inhibitors) and incubated on ice for 20 minutes. Following addition of NP-40 (0.2% final concentration) cells were further incubated 1 minute on ice and spun at 600g for 5 minutes. Supernatants were subsequently centrifuged at 13000 rpm for 15 min to pellet unbroken nuclei and organelles to obtain clear cytoplasmic lysates. Nuclei were briefly washed in hypotonic buffer to eliminate traces of detergent, purified on sucrose cushion, counted and lysed in RIPA buffer. To verify the purity of the subcellular fractionation, anti-PARP1, anti Histone H1, anti-rack1 and anti actin antibodies have been used in WB.

Proliferation Assay

For the CFE assay (colony formation efficiency assay) U2OS (1×10^5 cells per well) cells were seeded into 6-well multiplates, transfected with 2 μg of total plasmid DNA using the standard calcium phosphate method and processed as previously described [53]. Graphs (% of remaining colonies ± S.D.) represent the mean of at least four experiments. Statistical analysis of variance was performed as previously described.

p53 Stabilization Assays

Stabilization of p53 was performed in U2OS transfected with wt, T8A and T8D mutant plasmid constructs. Protein levels were analysed 24h after transfection with anti ARF (C-18), anti p53 (DO-1) and anti actin antibody as loading control.

Phosphorylation Assays

The MBP::ARF fusion proteins (both wt and mutants) were obtained as previously described [53] and subjected to an in vitro phosphorylation assay using a commercially available kit following manufacturer instructions (Stratagene). PP1 dephosphorylation assay was performed with the catalytic PP1 subunit (Promega) following manufacturer instructions.

Lambda-phosphatase (λ-PPase; New England Biolabs) treatment was performed as previously described [55]. Immunoprecipitation following TPA treatment: 4 mg of total cellular extracts of H1299 cells treated or not with TPA (10 μM for 2 min) were lysed in IP buffer (Tris-HCl pH7 10mM, NaCl 150 mM, SDS 0.1%, Triton-X100 1%, EDTA 1mM, EGTA 0.5mM) plus protein phosphatase inhibitors. Cleared lysates were incubated with 3 μg of anti ARF (Ab2 14PO2, Neomarkers) ON. After washing immunocomplexes were subjected to SDS-page on a 13% gel followed by incubation with anti pThreonine (1:100 dilution in 3%BSA) followed by chemiluminescence (Pierce). The filter was then stripped and incubated with anti ARF antibody (C-18) in order to visualize total immunoprecipitated ARF protein.

Co-Immunoprecipitation

ARF/pPKC co-immunoprecipitation assay were performed with 2 mg of total cellular extracts. Cells were harvested, lysed with co-ip buffer (Tris-HCl pH7 10mM, NaCl 150 mM, NP40 0.5%, glycerol 10%, EDTA 0.5mM) and incubated ON with anti ARF antibody (C-18 Santa Cruz). After washing immunocomplexes were subjected to WB and analysis with anti pPKC pan antibody (Beta IISer660, Cell Signalling), anti ARF (Ab2 14PO2, Neomarkers) antibody and anti MDM2 antibody.

ARF (wild type and mutants)/MDM2 or myc tagged p32 co-immunoprecipitation assays were performed with 1 mg of total cellular extracts. Twenty-four hours following transfection cells were harvested and processed as described. Ip were carried out with anti X-press antibody and immunoprecipitated samples

analysed by WB with anti MDM2, anti myc and anti ARF antibodies.

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Author Contributions

Conceived and designed the experiments: MV GLM RAC. Performed the experiments: MV FS CS MR. Analyzed the data: MV GLM RAC CS FS VC AP MR. Wrote the paper: MV GLM.

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